Genes, Oncogenes, and Hormones

# Cancer Treatment and Research

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# Genes, Oncogenes, and Hormones

## Advances in Cellular and Molecular Biology of Breast Cancer

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**Springer Science+Business Media, LLC**

**Library of Congress Cataloging-in-Publication Data**
Genes, oncogenes, and hormones: advances in cellular and molecular biology of breast cancer/Robert B. Dickson and Marc E. Lippman, eds.
p. cm. — (Cancer treatment and research; v. 61)
Includes bibliographical references and index.
ISBN 978-1-4613-6552-5 ISBN 978-1-4615-3500-3 (eBook)
DOI 10.1007/978-1-4615-3500-3
1. Breast — Cancer — Molecular aspects. 2. Cancer cells.
3. Oncogenes. 4. Steroid hormones — Receptors. I. Dickson, Robert B. (Robert Brent), 1952– . II. Lippman, Marc E., 1945– .
III. Series.
[DNLM: 1. Breast Neoplasms — genetics. 2. Oncogenes — genetics.
3. Receptors, Steroid — genetics. W1 CA693 v.61]
RC280.B8G45 1992
616.99'44907 — dc20
DNLM/DLC
for Library of Congress 92-15491
CIP


Originally published by Kluwer Academic Publishers in 1992
Softcover reprint of the hardcover 1st edition 1992


*Printed on acid-free paper*

# Dedication

## William L. McGuire, M.D.

As we and many of our colleagues were putting the finishing touches on our chapters for this volume, we were all shocked and deeply saddened to learn of the sudden death of Bill McGuire on March 22, 1992 in Cozumel, Mexico. This tragic event is an enormous personal loss for all of us and will deprive the scientific world of one of its brightest stars. His many remarkable accomplishments will be well known to those who would pick up this volume. Virtually every chapter in this volume reveals insights into Bill's far reaching contributions into every aspect of breast cancer biology. We do not believe it to be an exaggeration to say that his discoveries have directly resulted in an improved and prolonged life for many thousands of women afflicted with breast cancer. That legacy along with so many others will most surely endure. We offer our condolences to his family and to the many colleagues with whom he worked so closely over the years and whose careers owe so much his intellectual and personal gifts.

We wish to dedicate this volume to Bill's memory as a means of expressing our gratitude to him for all that he contributed to the field and as a tribute to our very close personal friendship. Moreover, we hope that this, our third volume devoted to the *Cellular and Molecular Biology of Breast Cancer*, will be only one of a series of scientific and literary efforts which will continue on the path that Bill has so ably illuminated until our task of ending the scourge of breast cancer is complete.

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# Cancer Treatment and Research

## Foreword

Where do you begin to look for a recent, authoritative article on the diagnosis or management of a particular malignancy? The few general oncology textbooks are generally out of date. Single papers in specialized journals are informative but seldom comprehensive; these are more often preliminary reports on a very limited number of patients. Certain general journals frequently publish good in-depth reviews of cancer topics, and published symposium lectures are often the best overviews available. Unfortunately, these reviews and supplements appear sporadically, and the reader can never be sure when a topic of special interest will be covered.

*Cancer Treatment and Research* is a series of authoritative volumes that aim to meet this need. It is an attempt to establish a critical mass of oncology literature covering virtually all oncology topics, revised frequently to keep the coverage up to date, and easily available on a single library shelf or by a single personal subscription.

We have approached the problem in the following fashion: first, by dividing the oncology literature into specific subdivisions such as lung cancer, genitourinary cancer, pediatric oncology, etc.; and second, by asking eminent authorities in each of these areas to edit a volume on the specific topic on an annual or biannual basis. Each topic and tumor type is covered in a volume appearing frequently and predictably, discussing current diagnosis, staging, markers, all forms of treatment modalities, basic biology, and more.

In *Cancer Treatment and Research*, we have an outstanding group of editors, each having made a major commitment to bring to this new series the very best literature in his or her field. Kluwer Academic Publishers has made an equally major commitment to the rapid publication of high-quality books and to worldwide distribution.

Where can you go to find quickly a recent authoritative article on any major oncology problem? We hope that *Cancer Treatment and Research* provides an answer.

William L. McGuire
Series Editor

# Preface

In *Requlatory Mechanisms in Breast Cancer*, our second volume in the *Advances in Cellular and Molecular Biology Series*, we tried to continue the themes begun in the first volume: oncogenes and antioncogenes, growth factors, steroids, and stromal-epithelial interactions. The past year has seen such advancements in all of these areas that we has no trouble in putting together the current volume along the same thematic lines. We hope the response to this third volume is as excellent as the response to the first two. We are particularly excited about the possibility that the new molecular understanding of regulation of growth, differentiation, and metastases will lead to better tumor prognosis and treatment. For this reason we have chosen to lead off the book with two excellent chapters in this area.

The first section is devoted to a better understanding of the biology of node-negative breast cancer and of the biology of the metastatic transition. The first chapter, by Elledge and McGuire, summarizes the rapidly emerging data on the field of prognostic factors in node-negative breast cancer. This chapter sets forth the realistic hope that soon physicians will be able to utilize molecular markers in clinical decisions concerning the mode of patient therapy.

The second chapter, by Stetler-Stevenson and coworkers, presents very new data on the structure and role of type IV collagenases in breast cancer. These are critical enzymes whose role must be understood if we are to make an impact on preventing and treating metastases.

The second section is on suppressor genes and negative growth factors in breast cancer. The first chapter of this section, by Steeg, presents an overview of this important area. This chapter also highlights the function of nm23, a new suppressor that may function at the level of metastases. The second chapter, by Fung and T'Ang, focuses on the retinoblastoma gene; this support gene appears to be lost during breast tumor progression. The third chapter, by Grosse and coworkers, highlights a new inhibiting growth factor made by the breast — MDG1. The final chapter of the section, by Wakefield and coworkers, deals with the well-known inhibitory factor TGFβ. This factor is emerging as central to the regulation of normal breast and the transition from normal to malignant.

The third section treats the topics of oncogenes and stimulatory growth factors. DiFiore and Kraus, in the first chapter, summarize much of the literature that supports a critical role for erbB/EGF receptor family of tyrosive kinase oncogenes in breast and other neoplasias. The second chapter, by Lofts and Gullick, continues this theme but focuses on the c-*erb*B2 oncogene itself in breast cancer. Measurements of levels of this oncogene are becoming critical to breast cancer diagnosis. The next chapter, by Paik and coworkers, continues the c-*erb*B2 theme with more data on role of this oncogene in tumor progression and the response to chemotherapy and hormonal therapy. The fourth chapter, by Park and coworkers, presents data supporting the possibility that c-*erb*B2 is a viable target for immunotherapy of breast cancer. The fifth chapter in this section, by Lehtola and coworkers, treats the signal-transduction mechanism of the c-*erb*B2 oncogene. A novel approach utilizing chimeric receptors is utilized. The sixth chapter of the section, by Fabbro and coworkers, continues the theme of signal transduction by tyrosine kinase protooncogenes. These investigators have focused on protein kinase C as an intermediary step in the cascade of intracellular events triggered by tyrosine kinase oncogenes. The final chapter of this section, by Dickson and coworkers, completes the discussion of signal transduction. This chapter is on the interaction of tyrosine kinases and nuclear protooncogene expression in breast cancer.

The fourth section deals with steroid hormone receptors. Since Beatson's original observations in the 1890s of ovarian influences on breast cancer growth, this has been a critical area of breast cancer research. The first chapter, by Read and Katzenellenbogen, deals with critical events in the regulation of estrogen and progesterone receptors and genes they regulate. The second chapter in this area, by Bettuzzi and coworkers, focuses on the structure of estrogen and progesterone receptors. The final chapter, by Beck and Edwards, goes into considerable depth concerning the structure and function of the progesterone receptor. Though estrogen was initially thought to be the principal ovarian hormone influencing normal and malignant breast, now many researchers are turning to progesterone for a more complete understanding of breast epithelial proliferation, differentiation, and metastases.

The last section of the book is devoted to the differentiation and function of breast cancer epithelium and stroma. The first chapter, by Taylor-Papadimitriou and coworkers, deals with intermediate filament proteins called keratins. Detailed knowledge of the expression of keratin subtypes is critical for understanding breast epithelial cell lineages, gene regulations, and pathological identification of metastases. The next chapter, by Greenberg and coworkers, focuses on casein gene expression in mammary epithelium. This gene is yielding clues valuable for understanding hormonal and tissue-specific aspects of gene expression in general. The fourth chapter of the section, by McKnight and coworkers, treats a second milk protein, the whey acidic protein. This gene is also yielding clues to detailed regulatory

pathways of gene expression in breast epithelium. Finally, the last chapter, by Cullen and Lippman, treats developing knowledge on interactions of stroma and epithelium in the regulation of normal and malignant breast. The profile of growth factors, proteases, and matrix components synthesized by stromal cells adjacent to breast tumor cells is emerging as potentially vital to tumor diagnosis and to an understanding of tumor regulation. This section brings us full circle to the themes begun in the first section. Node-negative breast cancer may be studied from the point of view of epithelial cell biology and pathology, or stromal cell biology and pathology.

We hope this volume will be useful to students, clinical researchers, and basic researchers on breast cancer. It is not meant to replace the first two volumes, but to stand with them as part of a useful set covering some of the diversity of exciting new findings in breast cancer.

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PART I

# The progression of node-negative to -positive breast cancer

# 1. Prognostic factors in axillary node-negative breast cancer

Richard M. Elledge and William L. McGuire

## 1. Introduction

Breast cancer will be diagnosed in approximately 150,000 women in the United States each year [1]. Decisions on how and whether to treat after local therapy will have a large impact in terms of women affected and health care dollars spent. It is important, therefore, that these decisions be based on a rational review of sound data.

Of women with breast cancer, 50% will be cured of their disease or sustain a long-term remission by surgery alone or surgery combined with radiotherapy. Differentiating this group from those who will recur is important, both in order to decrease the rate of recurrence in those with residual disease by treating with systemic adjuvant therapy and not to treat those already cured, who could not benefit, and may be harmed by therapy. Of those with metastasis to axillary nodes, the chance of recurrence is 50% at 5 years [2] and 80% at 10 years [3], and in light of this, most if not all would agree that systemic adjuvant treatment is warranted with few exceptions. Women without axillary node metastasis at the time of surgery have a much better prognosis, with 30% recurring at 10 years [4]. Several recent studies have demonstrated that this group, as a whole, has a prolonged disease-free survival, though no overall survival advantage when treated with adjuvant therapy [5,6,7,8]. From these data it could be argued that benefits outweigh risks and that all women with node-negative breast cancer should receive some form of systemic treatment [9,10]. In contrast, others assert that since no survival advantage exists, risks and cost do not justify treatment of the entire group [11].

In the following review the characteristics of breast tumors that are known to be associated with differences in recurrence or survival will be discussed. Emphasis will be placed on findings from studies done on node-negative women in which follow-up is the longest and statistical power is the greatest. In addition, different methods of decisionmaking using these characterisics will be presented.

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

## 2. Tumor size

Of all prognostic factors, tumor size is the simplest to measure and costs virtually nothing. It is a good enough indicator of stage of disease that it is one of the cornerstones of the TNM classification of cancer staging. Stage predicts prognosis for most solid tumors. For breast cancer, size alone predicts for recurrence, but in a way that is not linear or always clear cut.

In 1969, in a study involving 2578 patients [12], increasing size was found related to a greater likelihood of positive nodes. Also, as size increased, for all patients there was a trend towards a greater 5-year recurrence and mortality rate, with tumors of 1.0–1.9 cm resulting in a mortality rate of 22% and those of >6 cm resulting in a mortality rate of 55%. However, when only node-negative patients were looked at, there were smaller less significant differences seen, with survival being 85% at 5 years when tumors were 1.0–1.9 and 75% when tumors were greater than 6 cm. Another finding was that even very small tumors (<1.0 cm) had metastasized to axillary nodes in 22% of 52 cases. Valagussa [4], in a study of 716 patients, had similar findings in that relapse and survival at 5 and 10 years was correlated with the size of tumor if nodes were positive but not if they were negative. There was a trend towards better 5- and 10-year survival for women with $T_1$ tumors as opposed to $T_{3a}$ lesions, which may have become significant if sample size was larger. Fifty-two node-negative women with $T_1$ lesions had 5- and 10-year survival of 98.1% and 90.1%, respectively, compared with 84.5% and 81.3% for 29 women with $T_{3a}$ tumors.

Other studies show a stronger association between the size and outcome. In 1458 women who were followed for an average of 30 years, size correlated with survival for node-positive and node-negative women [13]. In an analysis of a series of 646 patients, those with tumors of >5 cm were more than 1.5 times more likely to recur than those with tumors of 2–5 cm, and though size correlated with lymph-node status, size was an independent predictor of recurrence in a Cox regression model [14].

Three large studies of node-negative patients found size to be important in prognosis. In one series of 1647 women, size was highly predictive of both disease-free survival ($p = .0002$) and overall survival ($p = .0041$) [15]. With 7649 node-negative women, Nemoto [16] found that the 5-year 'cure' rate steadily declined as tumor size increased. For patients with tumors of 1.1–2 cm, this was 68.4%, dropping to 56.8% for tumors >5 cm. Again, however, as described earlier [12], when looking at the extent of lymph node metastasis in relation to tumor size in all 15,000 women studied, it was striking that even very small tumors (<1 cm) had metastasized to axillary nodes 25% of the time. Data from the SEER Program [17] are presented on Table 1. Women with tumors >2 cm and negative lymph nodes had an excellent prognosis at 5 years, with over 96% alive, this declining to 82% if the tumor size was >5 cm. As found earlier, increasing tumor size increased the probability of metastasis to axillary lymph nodes, and once again, even

*Table 1.* Relationship of tumor size to survival in node-negative breast cancer, in 13,464 patients

| Size | No. of patients | 5-year survival (%) |
|---|---|---|
| <2.0 cm | 5728 | 96.3 |
| 2.0–5.0 cm | 6927 | 89.4 |
| >5 cm | 809 | 82.2 |

From Carter et al. [17], with permission.

for tumors <1 cm, lymph node metastasis were detected 15% of the time.

Overall, tumor size can be used as a crude predictor of whether a tumor will metastasize, but there are many inconsistencies. Large tumors are only moderately more likely to metastasize than smaller ones, an effect that does not increase proportionately with size. Moreover, as seen in several studies, very small tumors have metastasis evident at surgery approximately 25% of the time. The hypothesis that the greater size, the greater time a tumor has to produce metastasis does not explain this finding. Other factors must enter in, including differences in cell biology in the original clone or differences that develop during the progression of growth. It can be concluded, however, that women with very small tumors, <1 cm, and lymph nodes that are histologically negative have an excellent prognosis, with the probability of recurrence being 10–15%. Adjuvant therapy would be helpful to only a very few of this group.

## 3. Hormone receptors

The use of the estrogen receptor (ER) as a predictor of clinical course in primary breast cancer was reported in 1977 [18]. Since then, evidence has been conflicting, with some studies showing improved prognosis when ER protein was detectable in the tumor [19–21], while others did not find this improvement [22,23]. Differences may have been due to sample size, the population sampled, varying methodology in measuring and defining receptor protein levels, different follow-up periods, and whether or not adjuvant therapy was given.

Some have found that ER positivity is predictive of a relatively lower recurrence rate over an initial 2-year period [24], but because of a gradual accumulation of relapses, after 4 years no significant difference in longer term recurrence rate was found on the basis of the presence or absence of ER. Using menopausal status as an additional discriminant has brought conflicting results [24,25].

In a relatively large study from Sloan Kettering, 556 women who were axillary node negative were followed for a median of 6 years [22]. A total of 256 were ER positive and 233 were ER negative or ER 'borderline.' None

*Table 2.* Comparison of survival and disease-free survival of axillary node-negative women according to ER status

| | NSABP [28] | | | Cleveland [27] | | |
|---|---|---|---|---|---|---|
| | ER+ | ER− | p value | ER+ | ER− | p value |
| Patient number | 522 | 300 | | 378 | 132 | |
| 5-year recurrence free, % | 74 | 66 | $p \leq .001$ | 78 | 67 | $p < .03$ |
| 5-year survival, % | 92 | 82 | $p \leq .001$ | 92 | 75 | $p < .0003$ |

received any adjuvant therapy. There was no difference in survival between ER-positive and ER-negative patients (94% vs. 91%), nor in disease-free survival (85% vs. 86%). Recurrence rates in premenopausal and postmenopausal women were also similar regardless of receptor status.

Other series of larger or similar size show small but significant differences in prognosis when receptor status is considered. With 807 node-negative patients, the Danish Breast Cancer Cooperative Group [25] found a longer relapse-free survival over 50 months for women with ER-positive ($p = .07$) and PgR-positive ($p = .02$) tumors. When broken down by menopausal status, the presence or absence of receptors was predictive for premenopausal women only. In 1647 women with node-negative breast cancer collected in San Antonio, ER positivity was highly statistically significant in the prediction of disease-free survival ($p < .0001$) and survival ($p = .004$) [26].

Table 2 compares the results of two large series looking at survival and disease-free survival while comparing receptor status when nodes were negative. Both show small but significant advantages in recurrence and survival at 5 years in women whose tumors were ER positive. In the NSABP study survival was slightly improved in both premenopausal and postmenopausal women who were receptor positive [28]. This was not the case in the Cleveland study, where only postmenopausal, ER-positive women had a significant advantage in outcome in relation to ER-negative women [27]. Recurrences were greater for premenopausal ER-negative women for the first 2 years only but were equal thereafter.

For all patients in the NSABP study, PgR positivity was associated with an 8% increase in survival ($p = .002$) but no significant difference in disease-free survival. In the Cox model using ER, nuclear grade, PgR, and histologic grade, PgR was not independently predictive of survival. In contrast, PgR has been found by some to be a stronger predictor of relapse-free survival [25] and survival [29] than ER. Adjuvant endocrine therapy was given to some women in the later study and could have influenced the results. PgR was not measured in the Cleveland series.

Taken as whole, the data indicate that ER status predicts for small differences in recurrence and survival through 5 years, on the order of 8–12%. Over time, these differences lessen and may not exist as follow-up

becomes longer. This may be explained by the fact that ER-negative tumors recur sooner and then recurrence rate drops off, while ER-positive tumors recur less quickly but show a continued accumulation of recurrences over a longer period.

It is possible that in the future immunohistochemical assays for estrogen receptors could improve its clinical value [30].

## 4. Histopathologic findings

Breast cancer was first graded according to degree of aggressiveness in 1925. Since then many pathologic characteristics have been examined with contrasting results by different investigators. Tumor cell population is histologically heterogeneous [31] and sections evaluated account for only a small portion of the tumor. Evaluation of histologic type and grade can suffer from interobserver variability [32,33]. Despite these problems, several histopathologic findings from a number of studies can be consistently linked with clinical course to a firm enough degree to draw valid conclusions.

A widely used schema for assessing histologic and nuclear grade is one by Scarff–Bloom–Richardson (SBR) [34]. This schema assesses the degree of duct formation by tumor cells; the amount of variation in size, shape, and structure of the nuclei; and the number of mitoses per high-powered field. A score of 1–3 is given in each category. The total score is then summed. Grade I is 3–5, Grade II is 6 or 7, Grade III is 8 or 9, Grade I is the most differentiated, and Grade III is the least differentiated. The Ludwig Breast Cancer Group looked at 1537 node-positive women treated with adjuvant therapy and found 5-year survival at 86%, 70%, and 57% in Grade I, II, and III, respectively [35]. For all grades this was highly statistically significant.

A criticism of the SBR system is that 50–60% of patients cluster in Grade II, making no clear separation of good vs. bad prognosis for this larger portion. In an effort to eliminate the shortcoming, Doussal [36] created a modified SBR (MSBR) system by eliminating the evaluation of tubule formation and having five subdivisions based on nuclear pleomorphism and frequency of mitosis. This was based on the finding that tubule formation was the least predictive of the three SBR criteria when applied to 1262 women with invasive breast cancer. MSBR was more important than SBR in predicting disease-free survival in a multivariate comparison. For the 650 node-negative patients, this also was true [37] and, in addition, the MSBR grading system was significant in predicting the overall survival. Importantly, as seen in Table 3, more patients can now be placed into a low-risk group (MSBR Grade I, II, III), in which there were only six relapses out of 245 women (2.4%), and a high-risk group (MSBR Grade V), in which the relapse rate was much higher (19%). This occurred because of placement of some SBR II patients into a more defined higher or lower risk group. The

*Table 3.* Distribution of patients in SBR and MSBR groups and relative risk of metastasis

| | Number of patients (%) | Recurrence (% of each group) | Relative risk | |
|---|---|---|---|---|
| SBR grades | | | | |
| I | 87 (14) | 2 (2.3) | 1.0 | |
| II | 366 (56) | 24 (6.6) | 2.9 | p = .001 |
| III | 197 (30) | 30 (15.2) | 6.1 | |
| MSBR grades | | | | |
| 1 | 21 (3) | 0 (0) | — | |
| 2 | 72 (11) | 1 (1.4) | 1.0 | p = .00005 |
| 3 | 152 (23) | 5 (3.3) | 2.4 | |
| 4 | 263 (41) | 23 (8.7) | 5.9 | |
| 5 | 142 (22) | 27 (19.0) | 12.1 | |

From Le Doussal et al. [37], with permission.

number of women of intermediate risk (SBR Grade II or MSBR Grade IV) decreased from 366 (56%) to 263 (41%).

In other even larger trials involving node-negative women, nuclear grade was a strong predictor of survival [38,39] and has been judged to be better than receptor status by some [28] but not by others [40].

Histologic type can also be correlated with survival. In an NSABP study [38], a mucinous, tubular, or papillary appearance was most favorable, with not otherwise specified (NOS) or atypical medullary being least favorable, and typical medullary and lobular being intermediate in terms of survival. In part these different appearances are probably an indirect reflection of the level of differentiation — the more differentiated tumors tend to form identifiable structures.

Nuclear grade can be used as an important tool in assessing clinical outcome. There are significant differences in prognosis by this criteria for women with tumors of low vs. high nuclear grade, with good studies reflecting this. A caveat, however, is that the grading should be done by a pathologist who is experienced and follows standardized criteria. Histologic type is of value in that the relatively small percentage of tumors which display a mucinous, tubular, or papillary appearance are generally less aggressive in their behavior.

## 5. Cell kinetics and ploidy

Previously discussed factors can give indications of clinical outcome, though at times the associations are not strong. More direct measurements of the tumor cells' ability to divide and grow, along with determination of relative DNA quantity in each cell, have proven useful in predicting the course of node-negative breast cancer.

The thymidine labeling index is a method that measures the percent of

cells in S-phase over a given time, usually 30 minutes to a 2 hours. The proportion of nuclei labeled with tritiated thymidine corresponds to the proportion of cells in S-phase [41], and is termed the *labeling index*. An association between labeling index and clinical aggressiveness in breast cancer was reported in 1975 [42]. Over a 10-year period, 128 breast cancer patients from this original study were followed. Relapse-free survival and survival were significantly higher in women whose tumors had a low labelling index as opposed to a higher one [43]. Others have also reported an inverse correlation between the labeling index and time to relapse in women with breast cancer [44]. In 76 node-negative women who received no adjuvant chemotherapy, survival at 5 years was 84% vs. 100% for those with low vs. high labeling indices, respectively [45]. This grew more striking at 8 years, with all women alive whose tumors had a low labeling index, but only 36% surviving in the high-labeling index group. The difference in relapse-free survival was statistically significant. In a larger group of 258 node-negative women who received no adjuvant chemotherapy, relapse-free survival was 80% for women with slowly proliferating tumors vs. 60% for women with tumors of a higher proliferative rate [46]. Survival differences of 91% vs. 75% at 6 years for the two groups were also highly significant.

Thymidine labeling index suffers from the limitation of being time consuming and labor intensive. Thousands of cells must be manually and individually counted for each patient. With flow cytometry, the S-phase fraction can be computed for 10,000–100,000 cells quickly and easily. In addition, the ploidy status of the tumor cells can be derived. Finally, labeling index must be done on fresh specimens, while flow cytometry can be done on paraffin-embedded tissue [47]. Specimens from many years back can be retrieved from storage and analyzed.

Ploidy is technically easier to measure than S-phase, and several trials have shown its value in node-negative patients. The 8-year survival rate in a study of 165 women was 82% if the tumor was diploid, but only 63% if aneuploid [48]. Fallenins et al. also found nondiploidy to be associated with decreased survival over a 8- to 13-year period in 129 node-negative women [49]. Ellis noted a significant survival advantage at 5 and 10 years when women with euploid tumors (diploid plus tetraploid) were compared to those with aneuploid characteristics [50]. The euploid group, whose average age was 59.6 years, had a survival that was identical to the actuarial survival of 65-year-old women. Other investigators also noted differences in survival at 10 years according to ploidy, with a 33% death rate in 64 women with aneuploid tumors but only a 7% rate in 91 women if the tumor was diploid [51]. The authors concluded that ploidy was a powerful prognostic indicator in node-negative breast cancer patients. Not all have found this relationship, however. Rofagha discovered that essentially no differences in 5- and 10-year survival existed when 165 node-negative patients were compared on the basis of ploidy [52].

As technical ability has improved, the S-phase fraction can now be

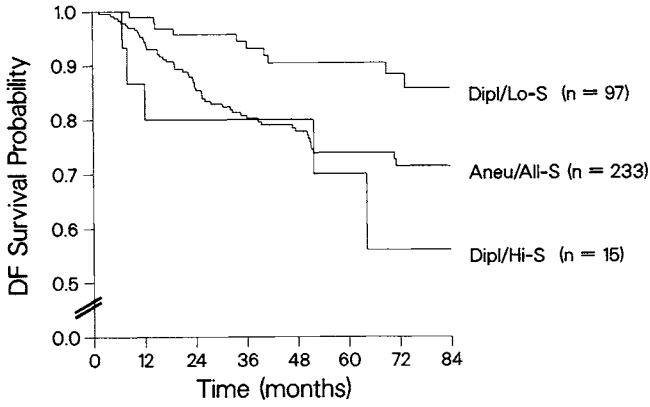


*Figure 1.* Comparative disease-free (DF) survival according to ploidy and S-phase fraction. (From Clark et al. [58], with permission.)

measured, along with ploidy, in a greater percentage of tumors. It has good correlation with the thymidine labeling index [53]. The findings of studies looking at both the S-phase and ploidy have varied. Ploidy was not related to recurrence or survival in 101 patients followed by Muss, though S-phase was of significance in predicting survival [54]. % S + % $G_2M$ was a stronger predictor than % S alone. With 20 years of follow-up, both S-phase and ploidy status trended towards, but did not reach, significance in predicting survival in 150 Finnish women with $T_{1-2}$ $N_0$ tumors [55]. In 541 node-negative patients followed over a median of 3.7 years, S-phase, but not ploidy, predicted the time to recurrence [56]. Reilly also found the S-phase to be more important than ploidy in predicting relapse-free survival at 5 years [57].

Clark et al. analyzed tissue from 395 tumors for S-phase fraction and ploidy. Data from this analysis are shown in Figure 1 [58]. Aneuploidy confered a lower disease-free survival at 5 years, regardless of the % S-phase. With diploid tumors, disease-free survival was different according to the S-phase fraction. If the fraction was low, disease-free survival was 90%, but this dropped to 70% if diploidy was accompanied by a high % S-phase. With 367 women, when dividing S-phase into two categories, high and low, Sigurdsson et al. [29] came to much the same conclusion as the former study. When, however, three categories of % S-phase were applied — low, intermediate, and high — the results changed in that % S-phase yielded more prognostic information than ploidy. Thus, by using a slightly different study design on the same group of patients, conclusions can seemingly be different.

Both ploidy and % S-phase can yield information that can be utilized in the clinical management of women with node-negative breast cancer. Those with tumors that are aneuploid or have a high S-phase fraction fall into a

category of relatively higher risk than women with tumors that are diploid and have a low S-phase fraction. As seen with other prognostic factors, standardization of methods is important to achieve consistent, reliable, and reproducible results.

## 6. HER-2/*neu* Proto-oncogene

The oncogene, *neu*, was first identified in transfection experiments as a point mutation in chemically induced rat neuroblastomas. The mutation resulted in the transformation [59]. *Neu* is also known as HER-2 or *erb*B2. The proto-oncogene is a membrane-bound 185-kDa receptor molecule with tyrosine kinase activity. It shares partial homology with the epidermal growth factor receptor erbB, but each gene lies on a separate chromosome [60]. A possible ligand for HER-2 was recently described [61].

HER-2/*neu* is not overexpressed in benign breast tissue [62]. Amplification of the HER-2/*neu* gene was first found in human breast cancer cells by King et al. [63]. In 1987 amplification of HER-2 was studied in a series of 189 women with breast cancer. At that time 2- to 30-fold amplification was found in 30% of cases and was related to decreased survival in women with positive nodes [64]. Unfortunately, no such relationship existed when lymph nodes were negative. A number of papers on the subject followed with conflicting results. Some could not confirm these original observations [65–68], while others have [69–73]. The methodology used in these studies varied widely, possibly contributing to their disparate results. Gene amplification and RNA and protein expression were examined with blotting procedures and immunohistochemistry alone, or in combination, depending on the study. Sample sizes and follow-up were different. Slamon et al. again examined gene amplification and protein overexpression, this time in over 500 patients, and reconfirmed their previous findings [74]. There was good, but not perfect, correlation between gene amplification and overexpression of the membrane protein. In another large series, Tandon did Western blot analysis on 728 patients and also concluded that those with positive nodes and higher levels of oncogene protein had shorter survival. Oncogene protein levels were not predictive of survival for node-negative patients [75].

While HER-2 status has not been able to predict survival in node-negative patients in most studies, a few have found a correlation. With 44 patients, data by Wright indicated a significantly better overall survival if patients' tumors stained negative for HER-2 [76]. After 7 years, in another group of 66 patients with negative nodes, those without gene amplification had a survival advantage [77].

Some have detected survival differences in small subsets of patients within the node-negative group based on HER-2/*neu* status and other defining characteristics. In an NSABP study involving 292 patients, node-negative women with good nuclear grade, as expected, did relatively well

[78]. However, when this subset was further broken down according to HER-2 expression, those with overexpression had a fivefold higher mortality. The actual number of overexpressors was so small that no firm conclusion can be drawn.

In 307 low-risk patients (ER positive, <3 cm, negative nodes) from a Cooperative Group study, HER-2 expression was analyzed along with histopathology. In a subset of patients consisting of those with purely invasive cancer (without a noninvasive component), HER-2 expression showed a strong correlation with early recurrence at 5 years and a decreased overall survival when compared with those without expression (C. Allred, unpublished data).

At the present time the role of the HER-2 oncogene in the clinical management of breast cancer is becoming clarified. It is generally agreed that increased amounts of HER-2 in women with positive axillary nodes predicts a decreased survival; however, all these women would normally receive adjuvant therapy regardless of their HER-2 status. Some would argue for even more aggressive treatment, such as autologous bone marrow transplantation. For most women with breast cancer that has not detectably spread to the axillary nodes at diagnosis, the assessment of HER-2 status is currently not helpful. Current research points to a small subset in which the knowledge of the amount of HER-2 may be useful but the total impact is slight.

## 7. Cathepsin D

Cathepsins are a group of hydrolase enzymes that can cleave interior peptide bands of various proteins. There are a number of different cathepsins, cathepsin D being but one of the group. Upon estrogen stimulation, the 52-kDa glycoprotein is secreted into the media of several breast cancer cell lines [79], and this protein has been characterized as a precursor of cathepsin D. About 40% of the precursor is secreted, while the remainder is accumulated in lysosomes as 48 kDa and 34 + 14 kDa mature products [80]. Here it performs its normal function in an acid environment of degrading endogenous proteins. Normal breast tissue contains low levels of cathepsin D [81], but secretion of cathepsin D is increased in breast cancer cells [82]. This has lead to the hypothesis that cathepsin D plays a role in carcinogenosis by digesting surrounding basement membrane and extracellular matrix, which is one step in the metastatic pathway. It is also a mitogenic agent that can directly stimulate the growth of estrogen-deprived MCF-7 cells [83]. How this occurs is not known, but it could function indirectly by cleaving growth factors from their precursors, freeing them from the extracellular matrix, or alternatively act directly by binding growth factor receptors. Thus, cathepsin D could be a promoter of metastasis, a stimulator of cancer cell growth, or both.

Several recent studies have looked at the possible relationship of cathepsin D, in its various forms, and prognosis in breast cancer. Thorpe assayed the 52-kDa form of cathepsin D in the cytosol of 396 breast tumors. Patients with high levels of the enzyme had a shorter recurrence-free survival than those with lower levels [84]. This was true in both lymph node-positive and lymph node-negative patients. There was no significant difference in survival according to cathepsin D levels, nor were levels associated with lymph node status, tumor size, or grade. Median follow-up was at least 48 months. Other studies have also found cathepsin D levels to predict the clinical course. In 122 patients followed for a median of 4.6 years, metastasis and disease-free survival were strongly correlated with cathepsin D concentrations, the association being stronger for the lymph node-negative subset [85]. Survival was actually worse in women with negative nodes and high cathepsin D levels than in women with positive nodes and low cathepsin D levels. Cathepsin D levels were independently prognostic from tumor size, grade, receptor status, and DNA index. Tandon measured the 34-kDa mature form of cathepsin D in 397 patients, 199 who were node-negative [86]. Overall, levels were higher in the group of node-positive women, indicating that cathepsin D may play a role in the ability of the tumor to metastasize to lymph nodes. As far as discrimination is concerned, however, higher levels of cathepsin D predicted shorter disease-free survival and overall survival for node-negative patients but not for node-positive ones. Figure 2 illustrates the relationship between levels of cathepsin D and the probability of survival. In node-negative patients in a multivariate analysis that included ploidy, progesterone and estrogen receptors, and tumor size, cathepsin D was the strongest predictor of prognosis in women with node-negative disease.

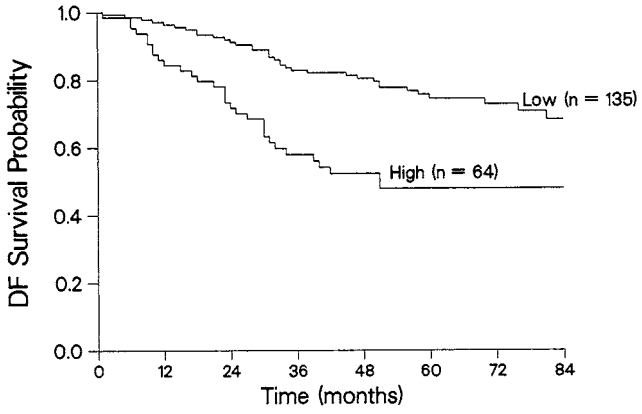


*Figure 2.* Disease-free (DF) survival in node-negative patients according to cathepsin D levels.

## 8. Methods of Decisionmaking with prognostic factors

Arriving at a decision using prognostic factor data can be accomplished using a number of approaches and techniques. These vary in their complexity and have different advantages and disadvantages.

The simplest approach is to assign patients into relative high-risk or low-risk categories based on a small cluster of prognostic variables. An example of this is classifying patients as high risk vs. low risk depending on whether their tumor was estrogen-receptor positive or negative, low or high S-phase, low or high cathepsin D, etc.

A more useful method is to use a rank-order bar chart, as discussed by McGuire et al. [88]. Probabilities of recurrence associated with the range of single or multiple factors are gained by a review of pertinent trials and studies in the literature. The factors are then put in order according to the absolute probability of recurrence. If a tumor has a mix of good and bad factors, the factor associated with the worst prognosis is used as the guide. Advantages are its ease of use and that probabilities of recurrences associated with different factors from diverse sources and studies can be pooled into one set.

Cox regression modeling takes a set of factors and mathematically compares their relative contributions in predicting outcomes after redundant information inherent between variables is eliminated. In this way interactions between variables are taken into account. The rank-order bar chart does not take these interactions into consideration. The problem with a Cox regression model is that new data from another set of factors from different studies cannot be introduced into the model. Sets with different factors or studies measuring disparate factors cannot be compared. At present, no study encompassing all factors together has been done.

Classification and regression tree analysis, also known as recursive partitioning, is a technique that can also account for interactions between variables. A tree is constructed with branches based on the variable with the strongest predictive power out of a group of variables after different cut points are evaluated, along with each factor's intrinsic predictive power. At each branch, a probability is derived for that set of factors, the set shrinking at each branch, until the final and least predictive factor is arrived at. By tracing the combination of factors associated with a given tumor through the tree, the branch that contains this combination is arrived at and the probability in that branch predicts the patient's recurrence.

Lastly, neural network analysis can be applied to prognostic factors. In this process, there are multiple simultaneous interactions and communications between pathways of information, much as there are multiple connections and interactions between a set of neurons in the central nervous system. The computer, rather than the programer, looks for relationships between variables; thus unexpected or unrealized relationships can be discovered without the knowledge or direction of the programer. The ability to

make complex comparisons and for the computer to independently recognize prognostic factor interactions is the main advantage over traditional modeling. Neural networks have been applied in making predictions about outcomes in breast cancer and the results are promising [89].

## 9. Conclusion

Aggressive breast cancer screening programs are becoming more prevalent and the use of mammography more widespread. A consequence of this will be that an ever-increasing number of women found to have breast cancer will be diagnosed at an earlier stage when lymph nodes are more likely to be free of disease. Determining and using tumor characteristics that predict recurrence, survival, and response to therapy in early-stage disease will thus become more important.

At present, data indicate that tumor size, estrogen-receptor status, histologic grade, ploidy, S-phase fraction, and cathepsin D can be used as indicators of the future clinical course of women with axillary node-negative disease. Other cell components of structure or function that are associated with tumor aggressiveness remain to be discovered.

Many questions remain unanswered and our present set of prognostic factors is not yet optimal. Because of this, it is important for physicians who care for women with breast cancer to encourage informed participation in clinical trials. Not only will patients receive the best 'state of the art' treatment, but questions can be answered and care can be improved for all those with breast cancer.

## Acknowledgments

This work was supported by NIH grant CA 30195. William L. McGuire, M.D. was a Clinical Research Professor of the American Cancer Society.

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# 2. Role of type IV collagenases in human breast cancer

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## 1. Introduction

In breast cancer, as in most other malignant human tumors, the most ominous aspect of the oncogenic process is the local invasion and subsequent metastasis of the primary tumor. The single most important prognostic factor in patients with breast cancer is the presence of neoplastic cells in the axillary lymph nodes [1]. If these nodes are positive for cancer cells, there is the potential for the dissemination of tumor cells to the lungs, adrenals, liver, bone, and brain. The growth of metastatic foci at these distant sites is the most common cause of death for patients with breast cancer. Although our understanding of the pathogenesis of metastasis formation has increased, progress has been slowed by the profound complexity of the metastatic cascade. One successful strategy for dealing with this complexity has been to separate the metastatic process into a series of steps and to define specific genes or gene products that are activated at each step [2]. This has led to the emergence of several basic themes, which have allowed identification of new strategies for the diagnosis and therapy of breast cancer.

It is now recognized that the development of breast cancer proceeds through a phase of uncontrolled cellular proliferation in situ to one of frank invasion, and that the loss of controlled cell growth does not in itself produce invasion and metastasis. The acquisition of the metastatic phenotype requires additional genetic changes over and above those necessary for unrestrained proliferation, allowing the tumorigenic and metastatic phenotypes to be distinguished [3–5]. The tumorigenic phenotype is characterized by localized cellular proliferation. In addition to the passive, uncontrolled growth seen in the tumorigenic phenotype, the metastatic subtype is dominated by a profound change in the interaction of the tumor cells with the extracellular matrix and the acquisition of actively incursive characteristics. Invasion and metastasis are characterized by tumor cell attachment to host cellular or extracellular matrix, augmented proteolysis that creates an extracellular matrix defect, and tumor cell migration through the resulting defect. The tumorigenic and metastatic phenotypes may be

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

facilitated by the action of gene products at a variety of levels, both intracellularly and extracellularly. However, it is now apparent that the attainment of these oncogenic phenotypes is the result not only of acquisition of positive effectors (oncogenes), but also loss of factors (antioncogenes) that block the production, action, or regulation of these effector genes. A common theme has emerged: In addition to the loss of growth control associated with the tumorigenic phenotype, tumor invasion and metastasis require an imbalance in the regulation of both cell motility and proteolysis, resulting in enhancement of both of these normal cellular functions.

Tumor cell interaction with the extracellular matrix, and in particular with the basement membrane, occurs at multiple stages in the metastatic cascade. General and widespread changes occur in the organization, distribution, and quantity of the surrounding epithelial basement membranes during the transition from carcinoma in situ to invasive carcinoma [6]. However, no matter how extensive the architectural disorganization, benign proliferative disorders are always characterized by a continuous basement membrane that surrounds the tumorigenic cells of the epithelial compartment and separates them from the stromal tissue. In contrast, invasive breast tumors possess defective basement membranes with zones of disruption and foci of complete loss of basement membrane components. Following its escape from the primary tumor, the metastatic tumor cell must interact with the subendothelial basement membrane during intravasation and extravasation from the vascular compartment during hematogeneous spread.

A general aspect of malignant neoplasms may be enhanced proteolysis of tissue barriers, such as basement membranes, favoring invasion [7]. However, proteolysis of such barriers is not unique to tumor cells. Extracellular matrix proteolysis also occurs during such physiologic processes as trophoblast implantation, mammary gland involution, embryo morphogenesis, wound healing, and tissue remodeling [8–10]. These processes, like tumor cell invasion, require both controlled proteolysis of the extracellular matrix as well as directed cell migration. The requirement for cyclic attachment to matrix components and subsequent release during the process of cell migration implies that the associated proteolytic events are well controlled and directed, possibly at the immediate cell surface of the invading tumor cells [11,12].

Enzymes from all four classes of proteases have been implicated in the process of breast-cancer invasion and metastasis (Table 1). All of these enzymes have been previously or subsequently identified in normal cells. Their association with the metastatic process is through their inappropriate overexpression in the invasive tumor cells. Invading tumor cells use normal enzymes in a temporally and spatially regulated fashion that resembles normal cells migrating through tissue barriers. Thus the difference in tumor cell invasion is that these processes, though coordinately regulated to allow tumor cell invasion, are deregulated with respect to normal cell function.

*Table 1.*

| Protease class | Substrate | Inhibitor |
|---|---|---|
| Cysteine<br>Cathepsin B<br>Cathepsin L | Extracellular matrix glycoproteins | Cystatins<br>Stefin A<br>Stefin B<br>Cystatin C<br>Kininogens |
| Aspartyl<br>Cathepsin D | Cell surface receptors ? mitogenic | ?<br>Pepstatins |
| Metal dependent<br>Collagenases<br>Type IV collageanses<br>Stromelysins | Collagens, glycoproteins (laminin, fibronectin) proteoglycans | Tissue inhibitor of metalloproteinases<br>TIMP-1<br>TIMP-2 |
| Serine<br>Urokinase<br>Plasminogen | Plasmin<br>Broad specificity | Serapins<br>PAI-1<br>PAI-2<br>Protease nexin |

Specific classes of endogenous inhibitors have been identified for most of these enzyme classes, and it is now apparent that the enzyme/inhibitor balance is the critical determinant of proteolysis. In fact, for the serine and metalloproteinases the use of inhibitors has provided direct evidence of a critical role for both of these enzymes in tumor cell invasion and metastasis formation [13,14]. This suggests that an enzyme cascade may be involved in the invasive process and that any one enzyme type may be necessary but not sufficient. However, inhibitors of the metalloproteinases, such as the collagenase family inhibitors like tissue inhibitors of metalloproteinases (TIMPs), have a demonstrated susceptibility to cleavage by serine proteases, such as macrophage elastase [15,16]. Similarly, inhibitors of serine proteases, such as $\alpha_1$-protease inhibitor, can be degraded and inactivated by matrix metalloproteinases, such as collagenases or gelatinases [17]. This suggests that, alternative to an enzyme cascade, the requirement for enzymes of both the serine and metalloproteinase classes for acquisition of the invasive phenotype might be the result of the synergistic action of these enzymes in removal of endogenous protease inhibitors, allowing further proteolysis of matrix components. The data on the role of the aspartyl and cysteinyl proteases in tumor invasion are primarily correlative, with little direct evidence for these types of enzymes in metastasis formation.

Among the list of enzymes involved in cancer, a large body of evidence has been accumulated regarding the collagenase family of metalloproteinases. The knowledge that the first barrier that most invading tumor cells must cross is the basement membrane, which contains type IV collagen as a major structural component, has focused attention on proteases that degrade type IV collagen. Two members of the collagenase family have demonstrated this ability, the 72-kDa type IV collagenase and the 92-kDa

type IV collagenase. The enzymes are named for their ability to degrade pepsinized, triple-helical type IV collagen. Two established features of these enzymes make their study particularly relevant to breast cancer. First is correlative evidence for enhanced expression of type IV collagenase in breast cancer. These results suggest that markers for these enzymes, and their associated inhibitors, may provide new diagnostic and prognostic indicators. Secondly, there is direct evidence that inhibitors of these enzymes block tumor cell invasion and angiogenesis, suggesting new therapeutic strategies. These lines of evidence will be discussed in following sections, but first we will discuss the levels at which type IV collagenase activity may be regulated, and we will review the role of type IV collagenases in the various stages of breast cancer.

## 2. The collagenase (matrix metalloproteinase) gene family

Nearly 30 years after the first identification of collagenolytic activity in the tadpole tail [18], the cloning and sequencing of individual genes has revealed a family of related proteinases with substrate specificity for structural extracellular proteins, such as the collagens [19–24]. The collagenase gene family can be divided into three subgroups based on substrate preference: interstitial collagenases, stromelysins, and type IV collagenases/gelatinases. The structural relationship between members of the collagenase family is shown schematically in Figure 1. Recently, new members of this family have been identified [25–27], promoter regions for several of the genes have been characterized [28–30], and new insights into the latency and activation of these enzymes have been gained [31–36].

Two members of the collagenase gene family are currently referred to as type IV collagenases. The term *type IV collagenase* has been used to describe the 72-kDa and 92-kDa metalloproteinase members of the collagenase family by a number of laboratories. Our use of this term is derived from a historical perspective and denotes the fact that the 72-kDa enzyme was first described as a tumor-associated protease activity that degraded basement membrane collagen [37–39]. These enzymes are distinct from other members of the collagenase family in two important respects. They possess a unique region immediately adjacent to the putative metal-atom-binding domain that shows close structural homology to the gelatin-binding domain of fibronectin (Figure 1). It has been suggested that this domain arises from exon shuffling between structural proteins, such as fibronectin, and these proteases [22]. This region is thought to be responsible for the affinity of these enzymes for denatured collagens. These two enzymes have been shown to specifically degrade type IV collagen to yield 1/4 N-terminal and 3/4 C-terminal fragments [22,23,39,40]. However, it is important to note that the 'physiologic' substrates for these proteinases are not established.

Indeed recent reports suggest that the 92-kDa enzyme may degrade type V collagen more effectively than type IV [40]. Both the 72-kDa and 92-kDa type IV collagenases are thought to possess elastinolytic activities, and the 72-kDa protease has demonstrated activity against type X collagen and fibronectin [41,42]. It appears that these enzymes may possess a much wider range of proteolytic activities than originally identified. Thus it may be impossible to determine the 'physiologic' substrate for these enzymes, and their 'physiologic action' may be dependent upon the spatial and temporal context in which the cell, normal or malignant, expresses a particular enzyme.

A second distinguishing feature of the type IV collagenases is their ability to interact, as latent proenzymes, with the tissue inhibitors of metalloproteinases (TIMPs) [43–45]. We have recently isolated, cloned, and sequenced a new tissue inhibitor of metalloproteinase, which we refer to as TIMP-2 [43,46]. Studies indicate that the interaction of these TIMPs with the latent type IV collagenases are selective and exclusive: The 72-kDa proenzyme forms a complex with TIMP-2 and the 92-kDa proenzyme forms a complex TIMP-1 [23,43]. The TIMPs do not appear to form complexes with other latent matrix metalloproteinases but will bind and inhibit all activated members of the collagenase family. Recent studies suggest that TIMP-2 may preferentially inhibit the 72-kDa type IV collagenase [47].

The type IV collagenases, like other members of the collagenase family, are secreted as latent proenzymes. This observation, along with the identification of specific latent proenzyme-inhibitor complexes, suggests that regulation of type IV collagenolytic activities may occur at many levels. These include translation/transcriptional regulation, activation of latent enzyme, and inhibition of active enzyme. Recent, important observations on each of these levels of type IV collagenase regulation are discussed in the next section.

## 3. Regulation of Type IV collagenolytic activity

### *3.1. Transcriptional and translational regulation*

Analysis of the transcriptional regulation of type IV collagenase expression has revealed that the 72-kDa type IV collagenase is regulated in a fundamentally unique manner when compared with other members of the collagenase family, including the 92-kDa enzyme [33]. This difference is best illustrated by the regulation of these genes by phorbol esters and transforming growth factor-β1 (TGF-β1). Exposure of cultured cells to phorbol ester has been shown to induce or enhance the expression of interstitial collagenase [33,48], stromelysin-1 [49], and the 92-kDa type IV collagenase

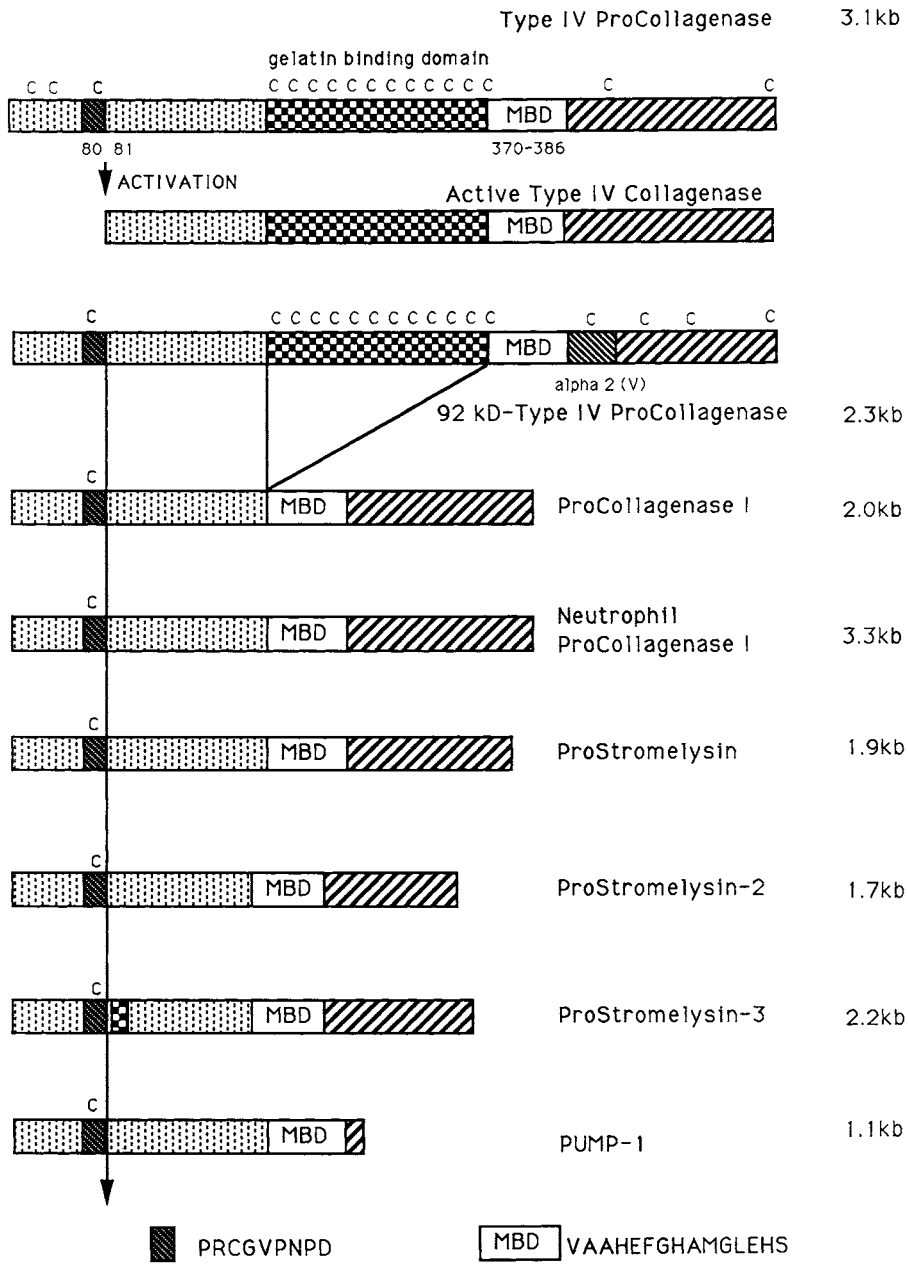


*Figure 1.* Collagenase gene family. Type IV procollagenase (72-kDa and 92-kDa forms), intersitial procollagenase, neutrophil procollagenase, prostromelysin, prostromelysin-2, prostromelysin-3, and PUMP-1 are represented diagramatically and aligned to show regions of protein sequence homology. MBD is the putative metal-binding domain. Type IV procollagenases contain a unique cysteine-enriched, putative substrate-binding domain that shows homology to fibronectin but that is absent from other collagenase family enzymes. Upon

[23]. This regulation is mediated, at least in part, at the transcriptional level and is consistent with the presence of a phorbol ester responsive element in the promoter region of the interstitial collagenase [28] and stromelysin genes [20]. In contrast, no such element has been detected in the promoter region of the 72-kDa type IV collagenase gene [29], and the expression of this enzyme is not induced or enhanced as a result of phorbol ester treatment of cells [33]. Similarly, treatment of cells with TGF-β1 decreases the expression of both interstitial collagenase [33,50] and stromelysin-1 [20]. Again, this effect appears to be mediated at the transcriptional level, as suggested by the discovery of a TGF-β1 inhibitory element (TIE) in the promoter region of the stromelysin-1 gene [30]. A TIE consensus sequence is also present in the interstitial collagenase gene, as well as other genes that are negatively regulated by TGF-β1 [30]. Such an element is not found in the sequenced regions of the 72-kDa type IV collagenase upstream element [29] and, consistent with this sequence information, the expression of the 72-kDa type IV collagenase is either unaffected or moderately upregulated by TGF-β1 treatment of human cell lines [33,51].

Although regulation by two agents represents only a small part of the overall picture of regulation in vivo, these studies serve to demonstrate how some members of the collagenase family may be coordinately regulated and expressed, and how others, specifically the 72-kDa type IV collagenase, may be expressed in an independent fashion. This observation may explain in part the apparent redundancy of the matrix metalloproteinase family, with several enzymes having similar or overlapping substrate profiles, such as stromelysin-1 and stromelysin-2, as well as the 72-kDa and the 92-kDa type IV collagenases. For the type IV collagenases, it is possible that the two enzymes are linked to different patterns of gene expression. These programs of expression may be developmentally based or may be discrete elements of processes such as wound healing and angiogenesis. The 92-kDa type IV collagenase may be coordinately expressed with other members of the collagenase family, although recent data suggest that these enzymes are also discoordinately regulated [52]. Further understanding of the roles of specific enzymes in the cancer-related processes of basement membrane destruction, interstitial matrix degradation, and angiogenesis will greatly aid in the identification of key targets for the development of new therapeutic strategies in breast cancer.

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treatment with organomercurial compounds in vitro, all eight enzymes are activated with concomitant removal of an N-terminal segement of 80–84 amino acids from the latent enzyme. This removed profragment contains an unpaired cysteine residue within the conserved amino acid sequence PRCGXPDV, located immediately adjacent to the proenzyme cleavage site. Site-directed mutagenesis studies have shown that alterations in this sequence result in spontaneous activation of transin, the rat homologue of stromelysin. In the latent proenzyme, this sequence interacts with the metal atom of the active site through the unpaired cysteinyl residue to block enzyme activity (see Figure 2). Pertubation of this cysteinyl residue-metal atom interaction results in activation of the proenzyme.

### *3.2. Activation of the latent type IV collagenase proenzyme*

Type IV collagenases, like other members of the collagenase family, are secreted by cells in culture as latent proenzymes. Unlike other members of the family, the 72-kDa and the 92-kDa type IV collagenases are complexed with TIMP-2 and TIMP-1, respectively [23,43]. The activation of these collagenases, therefore, constitutes an important, possibly unique, level of regulation. Recently, in vitro studies of the mechanism of activation of these enzymes has yielded new insights into the molecular basis of proenzyme latency [34–36]. The collagenase proenzymes can be activated in vitro by a variety of agents, including organomercurials, chaotropic agents, and other proteases. N-terminal sequence analysis of the 72-kDa type IV collagenase following organomercurial treatment revealed the loss of an 80-residue N-terminal domain [32]. Within this domain is a cysteine-containing sequence, PRCGXPDV, which is highly conserved among family members. Additionally, sequence comparison with other collagenases reveals that all proenzymes contain an odd number of cysteine residues [22,23,25,32, 34,35]. Titration of unpaired cysteinyl residues also reveals an absence of detectable free sulfhydryl groups in holoproenzyme preparations [36]. However, treatment of holoproenzyme with chelating agents to remove bound metal atoms resulted in detection of a single mole of free sulfhydryl per mole of enzyme [36]. This result suggests that the latency of the metalloproteinase is maintained by a specific metal atom-sulfhydryl side-chain interaction [34–36].

Collectively these data have been used to construct a hypothesis for the mechanism of activation (Figure 2). The latency of the proenzyme is maintained by an interaction between the zinc atom of the active site and the unpaired cysteine residue in the PRCGXPDV sequence of the profragment. Disruption of this interaction results in a conformational rearrangement, allowing attainment of collagenolytic activity [36]. The active proenzyme then undergoes autoproteolytic removal of the profragment, generating a stable active enzyme species. In the case of the 72-kDa type IV collagenase, the active enzyme generated by organomercurial treatment has a molecular weight of 62 kDa. Variations on this scheme, with minor changes, have been referred to as the 'velcro' mechanism or the 'cysteine switch' hypothesis [34,35].

To date, the insights gained into the mechanism of activation by these agents in vitro has not led to an understanding of the cellular activation of the 72-kDa type IV collagenase in vivo. Proteases, specifically plasmin, have been shown to be responsible for the activation of both interstitial procollagenase and prostromelysin-1 in cocultures of keratinocytes and dermal fibroblasts [31]. Furthermore, stromelysin-1 has been shown to activate interstitial collagenase, suggesting a proteolytic cascade in the regulation of interstitial collagenase activity [53,54]. However, plasmin and a variety of other proteases tested do not activate the 72-kDa type IV collagenase. More

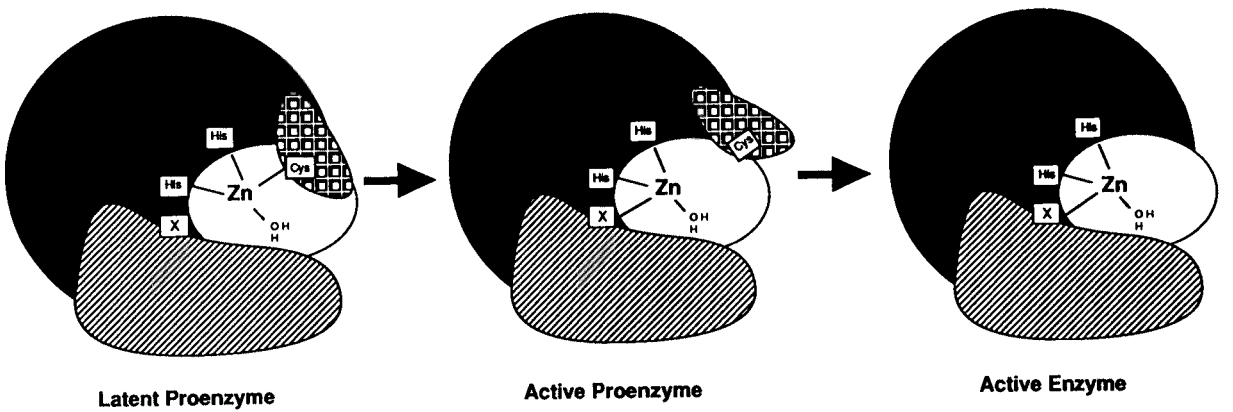


*Figure 2.* Model of type IV collagenase enzyme latent state and enzyme activation. Latency of the proenzyme is maintained by an interaction between the unpaired cysteinyl residue (CYS-73) of the profragment and the zinc atom of the active size. Disruption of this interaction results in the rapid attainment of full collagenolytic activity following a conformational rearrangement. The zinc atom coordination site contains two histidyl residues and shows homology with thermolysin. The third coordination position of the active enzyme is an as yet unidentified amino acid residue (X). Following conformational rearrangement and attainment of proteolytic activity, the active enzyme undergoes autoproteolytic removal of the N-terminal profragment to generate a stable active enzyme species.

recently, treatment of HT-1080 fibrosarcoma cells with phorbol ester or treatment of fibroblasts with concanavalin A has been shown to induce processing of the 72-kDa type IV collagenase to a 62-kDa activated form [33,56]. Analysis of the conditioned media from concanavalin A-treated gingival fibroblasts reveals a marked increase in type IV collagenase activity in the absence of organomercurial treatment [55]. The activation locus and N-terminal sequence of the final cellular-activated species are identical to those of the organomercurially activated form reported previously. Treatment with phorbol ester or concanavalin A, therefore, induces cellular activation of 72-kDa type IV procollagenase. Further studies have revealed that the mechanism for activation in each case appears to be the same. Metabolically radiolabeled 72-kDa type IV proenzyme/TIMP-2 complex was purified by gelatin-sepharose affinity chromatography and used as a probe for activation (Figure 3). The activity responsible for processing the latent complex to active enzyme was found to be confined to the cell monolayer and was not secreted into the soluble fraction. When phorbol ester or concanavalin A-treated cells were fractionated in situ using 0.2% (w/v) Brij-35, all of the activity remained associated with the residual cell fraction. The activity could be partially recovered from this fraction by extraction with higher concentrations of nonionic detergent, but not by high salt extraction [56]. This cellular activation of 72-kDa type IV collagenase appears to be a cell-surface-associated event. Inhibitor studies, using the residual fraction to activate the radiolabeled latent complex, revealed the cellular activation mechanism to be sensitive to inhibition by chelating agents, such as 1,10-phenanthroline and ethylenediaminetetraacetic acid, but insensitive to serine or thiol protease inhibitors. Additional exogenous TIMP-2 also blocked cellular activation of 72-kDa type IV collagenase. Although these inhibitor studies indicate that the mechanism is not plasmin mediated, it has not been possible to determine whether another metalloproteinase is responsible for activation or whether activation of the proenzyme is autocatalytic following a conformational change induced by interaction with a specific binding protein. The activation mechanism described is specific for the 72-kDa type IV collagenase [56], and although activated forms of the 92-kDa type IV collagenase have been observed in more complex organ culture systems [57], there has been no characterization of a cellular activation mechanism for this enzyme.

The existence of a plasmin-independent, cell-surface mechanism for the specific activation of a single collagenase family enzyme has important physiological implications. Such a mechanism would give a cell great control over the degradation of matrix in its immediate vicinity. With respect to tumor invasion and metastasis, it might allow a cell that expresses the 'activator' but not 72-kDa type IV collagenase to activate and utilize exogenous type IV procollagenase in tissues such as brain and bone [58,59]. The identification of the molecular species responsible for the cellular activation of the 72-kDa type IV procollagenase and other procollagenases

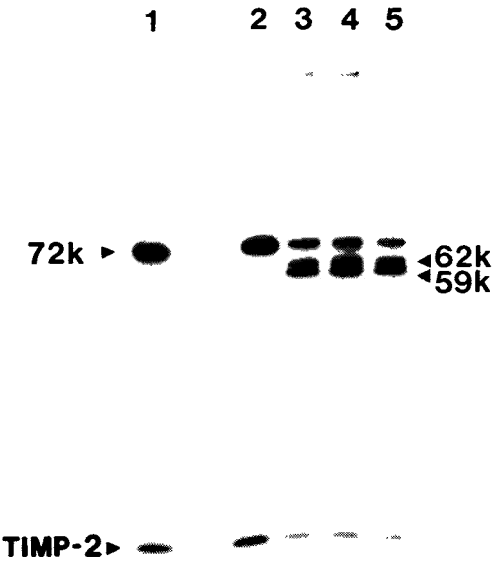


*Figure 3.* Cellular activation of exogenous 72-kDa type IV procollagenase/TIMP-2 complex. Affinity-purified, biosynthetically $^{35}$[S]-methionine-radiolabeled, 72-kDa type IV procollagenase/TIMP-2 was added to HT-10180 fibrosarcoma cells (30,000 cpm/ml) incubated in the presence or absence of phorbol ester (TPA, 10 ng/ml). After 24 hours, the conditioned media was collected and analyzed by polyacrylamide gradient gel electrophoresis, which was then processed for fluorography. The resulting autoradiograph is presented. Lane 1, affinity-purified, radiolabeled 72-kDa type IV procollagenase/TIMP-2 complex; lane 2, conditioned media from untreated cells; lane 3, conditioned media from phorbol-ester-treated cells (note that latent enzyme complex has been processed to lower molecular weight species, which are enzymatically active); lane 4, same as lane 3 in the presence of 200 μg/ml aprotinin; lane 5, same as lane 3 in the presence of 50 μg/ml of ε-amino-N-caprioc acid. These results demonstrate that activation of the 72-kDa type IV procollagenase/TIMP-2 complex is not dependent on serine protease or plasmin activity.

is likely to yield an important new series of target molecules in the development of treatments for invasive and degradative diseases.

### *3.3. Inhibition of type IV collagenase*

A third and apparently critical level of regulation of type IV collagenase activity is the inhibition of active enzyme by the TIMP family of proteins. Although the purpose and fate of the TIMP molecules in the latent type IV collagenase complex is unclear, it is known that once activated both type IV collagenases are susceptible to inhibition by either TIMP-1 or TIMP-2 [22,23,33], apparently preferentially by TIMP-2 [47]. TIMP-1 is a glycoprotein with an apparent molecular mass of 28.5 kDa. It was the first member of the TIMP family to be identified and has been cloned, sequenced,

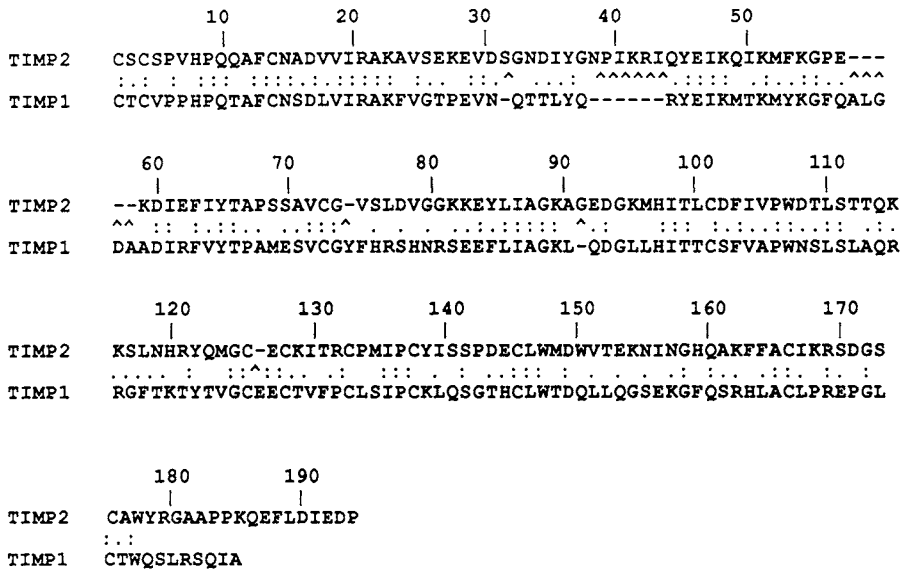


*Figure 4.* Comparison of TIMP-2 deduced amino acid sequence and TIMP-1 amino acid sequence. The homology of these two proteins is 38% identity with 66% similarity. The positions of all 12 cysteine residues are conserved, as are the positions of 3 of 4 tryptophan residues. This suggests that these residues are critical for the biological activity of these metalloprotease inhibitors.

and mapped to the 11p region of the X chromosome [60–62]. The secreted protein has 184 amino acids and six intramolecular disulfide bonds, whose assignment has recently been reported [65]. TIMP-1 is secreted primarily in uncomplexed form, frequently by cells that secrete interstitial collagenase [51].

TIMP-2, a second member of the TIMP family, was recently isolated, purified, and cloned [43,46,66]. TIMP-2 is a nonglycosylated protein with a molecular size of 21 kDa. Primary structure determination revealed a protein of 194 amino acids, showing 37% identity and 65.6% homology with TIMP-1 [46]. The positions of all 12 cysteines are conserved with respect to those present in TIMP-1, as are 3 of the 4 tryptophan residues (Figure 4). TIMP-2 is secreted as a complex with the latent 72-kDa type IV procollagenase and does not appear to be secreted in an uncomplexed form by the cultured cell lines tested [67].

Northern blot analysis has revealed the existence of two *timp*-2 transcripts of 3.5 and 1.0 kB [46]. cDNA probes to *timp*-2 are specific and do not detect the single 0.9 kB *timp*-1 mRNA species. These studies have also demonstrated that while TGF-β1 upregulates *timp*-1 mRNA expression [46,50,51], *timp*-2 mRNA and protein expression are downregulated in several cell lines following treatment with this cytokine [46,47]. Therefore, as with members of the collagenase family, the TIMPs are regulated independently. The

pattern of expression and specific roles for these inhibitors in vivo remains an area of active investigation.

## 4. Type IV collagenases in the progression of breast cancer

### *4.1. Evidence for the presence of type IV collagenase in human breast tumors*

The description of the regulation of type IV collagenases from studies in vitro clearly illustrates the steps required for type IV collagenase activity to be expressed in vivo. Following transcriptional activation and translation, the 72-kDa type IV collagenase is secreted in the latent proenzyme form complexed with TIMP-2. It is only when the proenzyme is activated and the levels of activated enzyme exceed the local concentrations of TIMPs that type IV collagenolytic activity is expressed. This may occur by localized production of active type IV collagenases at a sequestered site as a result of a cellular activation process or [31,56], as recently reported, the down-regulation of TIMPs [68].

There is evidence from several studies of collagenase and type IV collagenase production by both breast carcinoma cell lines and by breast carcinoma in situ. Collagenase activity has been detected in several human breast carcinoma cell lines [69], in mouse primary adenocarcinoma cell cultures, and in tumor-associated stromal cell cultures [70]. Primary tumor capsule cultures produced very little collagenase activity. It was also noted that collagenase production declined with time in culture. Type IV collagenase has been detected by immunohistochemistry in sections from human invasive breast carcinoma [71,72].

Several features of the type IV collagenase immunohistochemistry studies support the role of type IV collagenase in the progression of breast cancer. In an earlier study by Barsky et al. [71], intense immunoreactivity was only detected in invasive carcinomas, and carcinomas in situ and adjacent benign ductal structures were negative. Cases of fibroadenoma and fibrocystic disease also showed no immunoreactivity. In a more recent study [72], using affinity-purified antipeptide antibodies, cytoplasmic immunoreactivity for type IV collagenase was shown to be confined to myoepithelial cells in normal breast tissue, which were readily distinguished from the benign ductal epithelial cells. In progression from normal epithelium to carcinoma in situ, there is an increase in type IV collagenase immunoreactivity in the ductal epithelial cells, and in atypical hyperplasia the demarcation between the atypical ductal cells and the myoepithelial cells is lost. Of the invasive carcinoma examined, 36 out of 40 were positive for type IV collagenase, as were 10 out of 12 cases of metastatic breast cancer cells in the axillary lymph nodes [72]. The intracellular accumulation of this enzyme prior to actual invasion by the tumor cells is consistent with the hypothesis that the production or secretion of a latent enzyme alone is not sufficient for tumor

cell invasion. Therefore, a more critical determinant for invasion may be production of the cellular activator for the 72-kDa type IV collagenase following the upregulation of latent enzyme production. However, D'Errico et al. have shown that the number of cells staining positive in the primary tumor is greatly increased in both invasive ductal and invasive lobular carcinomas when compared with the in situ carcinomas [73].

Immunohistochemical staining for the 72-kDa type IV collagenase has been shown to be an effective prognostic indicator for local recurrence of disease [74]. However, no statistically significant difference in the rate of distant metastasis formation or overall patient survival was noted. These findings are consistent with the multistep nature of the metastatic cascade and indicate that the acquisition of the invasive phenotype through expression of type IV collagenolytic activity is necessary for local invasion but not sufficient for disseminated metastasis formation. In a separate study of interstitial collagenase activity in human breast, collagenase activity was found to be significantly higher in fibroadenomas than in benign cystic disease [75]. Levels in breast carcinomas exhibited a wide range of levels, encompassing levels observed in benign tissue. This suggests that elevated levels of interstitial collagenase are not an integral element of invasive breast cancer. Recently, a gene coding for a putative enzyme, referred to as stromelysin-3 [27], has been cloned and sequenced from human breast carcinoma tissues. Subsequent experiments demonstrated that the steady-state mRNA levels for this putative protease were present only in stromal fibroblasts adjacent to the edge of tumor cell invasion. Stromelysin-3 transcripts were present only in patients with invasive carcinomas and were not detected in patients with in situ carcinoma [27]. This suggests that tumor cell interaction with the surrounding stromal cells is another important level in the regulation of tumor-associated matrix proteolysis. Further characterization of the role of stromelysin-3 in breast cancer awaits identification of the protein, determination of enzyme activity and substrate specificity, and a more extensive correlation with other prognostic indicators.

### *4.2. Type IV collagenase and tumor progression*

Type IV collagenases and other collagenase family enzymes could potentially facilitate tumor progression in several ways. Obviously these proteinases may act to allow direct expansion of the primary tumor and metastasis, as they are likely to be involved in the invasion and migration of malignant tumor cells. In addition, these enzymes may play a role in the migration and invasion of endothelial cells during the process of tumor neovascularization.

Several studies have demonstrated the ability of tumor cells to degrade extracellular matrix in vitro. The varying degradation of subendothelial matrix by mammary adenocarcinoma cell lines was directly correlated with the level of type IV collagenase expression by these cell lines. This level was in turn correlated with metastatic potential as measured by experimental

lung colonization [76]. Other studies have also shown a positive correlation between type IV collagen degrading activity and metastatic potential [77]. The transfection of rat embryonic fibroblasts with the *ras* oncogene resulted in a marked increase in type IV collagenolytic activity secreted by these cells. Although the enzyme responsible for this activity was not characterized, the type IV collagenolytic activity was positively correlated with the number of experimental metastases [4].

The TIMPs have been used to demonstrate a direct role for the collagenase enzymes in tumor invasion. Studies have demonstrated that excess TIMP-1 and recombinant TIMP-1 (rTIMP-1) can block the invasion of human amniotic membranes by B16 murine melanoma cells and lung colonization by these same cells [14,78]. Recent studies have also shown that TIMP-2 [79] and recombinant TIMP-2 [80] can block tumor cell invasion in in vitro assays of cellular invasiveness.

## 5. Future directions

Investigations on the role of proteases in tumor invasion and metastasis have developed rapidly in the last 5 years. These studies have extended the initial correlation between collagenase enzyme expression and tumor progression to demonstrate a direct causal role for these enzymes in tumor invasion and metastasis formation. However, strong correlations between breast cancer progression and members of other protease families have recently been demonstrated. These include urokinase, as well as acidic lysosomal proteases, such as the cathepsins D, B, and L [80–82]. In the future an understanding of the cooperation between or synergistic action of members of different protease families may allow the identification of protease cascades involved in tumor invasion and metastasis formation. This would allow identification of critical points in the control of tumor-cell-mediated proteolysis and potential sites for therapeutic intervention. Future directions also include an understanding of the relative contributions of members within the same protease class. For example, it will be important to determine if the cysteine proteases cathepsin B and L have similar substrate specificities, and whether they contribute independently or interchangeably to the invasive phenotype. Also, it will be necessary to determine if enzymes with similar substrate specificities in vitro, such as the 72-kDa and 92-kDa type IV collagenases, contribute equally to the metastatic potential of invading breast cancer cells.

The demonstration that tumor cells produce both proteases and protease inhibitors suggests that the net balance of active protease species and protease inhibitors is critical in determining tumor cell-mediated proteolysis. Excessive proteolysis may effectively decrease tumor cell invasion and metastasis. This is because tumor cell invasion requires successive attachment and detachment of the migrating cells. Uncontrolled degradation

would remove the matrix scaffolding necessary for proper cell traction. Thus the invading tumor-cell-mediated proteolysis probably occurs in a highly directed manner and is tightly regulated. An understanding of the components of this regulation, including proenzyme activation, would allow targeting of these critical points in cell invasion.

The findings summarized in this report have generated much interest in the potential clinical application of native and synthetic protease inhibitors, particularly collagenase inhibitors. It is likely that research directed towards the clinical use of collagenase inhibitors, such as the TIMPs, will yield a more detailed picture of the role of these enzymes in diseases such as breast cancer. The pronounced similarities between the processes of tumor invasion and tumor-induced angiogenesis suggest that these collagenase inhibitors may have a dual role in the prevention of breast cancer progression. The observation that TIMP-1 and TIMP-2 inhibit angiogenesis suggest that these inhibitors may also prevent tumor growth, which is dependent on tumor-induced angiogenesis [83,84]. Theoretically, if administered at an early point in the disease process, these inhibitors could block the escape of tumor cells from the primary tumor and prevent metastasis formation. However, the limited practicality of this type of therapy in the clinical setting is realized, and the hope is that inhibitors such as TIMP-2 may act as tumor-static agents and prevent the local invasion and angiogenesis-dependent growth of both the primary tumor and established metastases. The use of collagenase inhibitors, such as TIMP-2, to block local invasion in established bone metastasis in patients with metastatic breast cancer should allow an initial assessment of the utility of this therapy in alleviating breast cancer morbidity.

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PART II

# Suppressor genes and negative growth factors

# 3. Suppressor genes in breast cancer: An overview

Patricia S. Steeg

## 1. Introduction

Over the past 10 years a multitude of oncogenes have been discovered, and considerable progress has been made in the characterization of their biochemical functions/malfunctions [reviewed in 1]. Cell-fusion studies have predicted the existence of suppressor genes for both the tumorigenic and metastatic components of cancer progression [2–4]. The general location of many putative suppressor genes was identified on the basis of chromosomal alterations, usually deletions, often in cancer types with a high risk of inheritance. One characteristic of many suppressor genes postulated to date is their homozygous inactivation in cancer cells, by deletion and/or mutation, consistent with Knudson's hypothesis [5]. Additional data, including experiments described herein for breast cancer, suggest that structural and/or regulatory alterations to suppressor genes, in the absence of homozygous inactivation, may also impact the cancer phenotype. Transfection experiments, however, remain the only accepted confirmation of a suppressive function. In these experiments the suppressor gene is transfected with a marker gene (usually antibiotic resistance) into tumor cells, and changes to a more 'normal' phenotype are noted. These experiments are fraught with difficulties, making their interpretation difficult: Often, transfection of the suppressor gene construct generates significantly fewer antibiotic-resistant colonies than a side-by-side transfection with a control construct. For *p53*, many of the transfected colonies that grew had *p53* mutations [6]. Although there are other possible interpretations, the data suggest that those tumor cells expressing the transfected suppressor gene and exhibiting a more 'normal' phenotype do not readily grow out as a colony. This may be due to direct effects on cell division or other changes in differentiation, contact inhibition, senescence, or viability. In no case can it be established that a transfected clone expressing the wild-type suppressor gene, and exhibiting a more 'normal' phenotype, did not concurrently require mutation at another chromosomal locus. The difficulty in obtaining stable transfected clonal cell lines expressing the wild-type suppressor gene

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

dictates that transfection experiments be reported in only a few tumor cell types.

## 2. Somatic allelic deletion

Somatic allelic deletion, the deletion of one allele of a genetic locus from tumor cell chromosomal DNA but not normal tissue, is a genetic alteration widely used to indicate the presence of putative suppressor genes. In practice these experiments are relatively simple: A RFLP (restriction fragment length polymorphism) must be identified for the probe to be used in allelic deletion studies. DNA samples from normal tissues of a series of individuals are cleaved with a variety of restriction endonucleases. Southern blots are then prepared and hybridized to the probe in question. Hybridizing bands in an allelic pattern are noted, i.e., two bands of different sizes, each exclusively present in some individuals (homozygotes) and both present in some individuals (heterozygotes). The restriction enzyme generating an allelic pattern on Southern blots is then used to digest DNA samples from matched normal and tumor tissue. Allelic deletion is quantitated only in individuals heterozygous for the probe, due to the difficulty in accurately determining the loss of one half of a hybridization signal for a homozygote. The deleted band is often greatly reduced in intensity in the tumor DNA sample but is not absent. This may be due to heterogeneity in the tumor cells or the presence of normal cells in the tumor specimen. All allelic deletion studies should compare the deletion rate to a background level, i.e., how often are randomly chosen genes, not thought to be suppressor genes, deleted in cancer cells? For example, Cropp et al. [7] reported allelic deletion at 7% of informative (heterozygous) cases for four background markers (*TGFA*, 2p13; *MYB*, 6q22–23; *PDGFB*, 22q12.3–13.1; somatostatin, 3p28) in a breast cancer patient cohort. Finally, allelic deletion studies often use genomic probes, not themselves thought to be a suppressor gene. In some cases, putative suppressor genes have already been localized to a region discovered to contain frequent deletions in breast tumors. In the other cases, identification of the putative suppressor gene is exceedingly difficult, often involving 'walking' the chromosome region to identify transcribed genes, and then investigating each gene for alterations in expression and/or structure in tumor cells. This type of analysis is made more difficult by the fact that allelic deletions often encompass large portions of the chromosome.

### *2.1. Chromosome 1*

Karyotypic analysis of breast tumor specimens have identified both the trisomy and structural rearrangements with breaks in chromosome 1 [8,9].

Hybridization studies for chromosome 1 loci have also noted multiple types of alterations, ranging from allelic deletion to gene amplification. Furthermore, the site and prognostic significance of allelic deletion on chromosome 1 varies significantly between reports. The data suggest the proximal localization of both oncogenes and suppressor genes, and/or multiple regulatory mechanisms for suppressor genes in breast cancer. No specific candidate suppressor genes have been identified to date on chromosome 1.

Allelic deletion studies at genomic loci on both 1p and 1q were reported by Genuardi et al. [10]. Allelic deletion of probes localizing to the 1p34–p36 region was reported in 41% of informative (heterozygous) cases. A reduced rate of allelic deletion was observed at other chromosome 1 loci, indicating that the results were not due to loss of a complete chromosome 1. The authors correlated allelic deletion using the D1Z2 probe at 1p36 with family history of breast cancer, early diagnosis of breast cancer, and the presence of multiple tumors or tumor foci. Allelic deletion at this locus did not correlate with any standard histopathologic criteria. Bieche et al. [11] noted an allelic deletion rate of 37% for the *L-myc* locus at 1p32. The authors did not map allelic deletion frequencies at probes more distal to *L-myc*, and it is therefore possible that the target of this deletion event is the same gene or genes identified at 1p34–p36 in the Genuardi study [10].

Chen et al. [12] noted allelic deletion at 23% of informative cases at a 1q21 locus, 23% at a 1q23 locus, and 26% at a 1q23–32 locus, as compared to a background rate of 8% allelic deletion in their cohort. No significant correlation was observed between 1q allelic deletion and patient age, as in the study at chromosome 1p. A significant association was observed between 1q allelic deletion and both the presence of lymph node metastases and estrogen receptor positivity ($p < .05$), with one marker indicative of poor prognosis and the other marker indicative of favorable prognosis. In apparent contrast to the Gunuardi study [10], the authors observed 0–13% allelic deletion using two probes directed to 1p31-pter.

Devilee et al. [13] examined allelic deletion at seven loci on 1p and 1q, and observed both allelic deletion as well as increased hybridization signals to one allele in tumor DNA samples relative to DNA extracted from matched normal tissue (termed *amplification* herein). Using a single probe, allelic deletion was observed in one breast tumor and amplification in another.

### *2.2. Chromosome 3*

In contrast to the situation on chromosome 1, there is a general agreement on the location and significance of allelic deletion on chromosome 3 in breast cancer. Devilee et al. [14] reported 54% allelic deletion rate at 3p21 and a 47% allelic deletion rate at 3p14–21 [15] in separate studies. Ali et al. [16] identified the 3p21–p25 region as the most common site for allelic deletion and suggested that the *c-erbAB* and *c-erbA2* hormone receptor genes may be targets.

### 2.3. *Chromosome 11*

Allelic deletion at 11p15.5 was reported by Devilee et al. in 19% of informative breast cases and 21% of informative cases [14,15] in two separate studies. Loci on chromosome 11p13 and 11p15 have been implicated in a syndrome exhibiting congenital absence of the iris of both eyes, genitourinary abnormalities in males, and the tendency to develop Wilm's tumor. A candidate gene, mapping to 11p13, encodes a zinc finger protein thought to be a transcription factor, but surprisingly it is expressed in many Wilm's tumors [17].

### 2.4. *Chromosome 13*

Mapping of allelic deletion rates at multiple loci along chromosome 13 in breast tumors was reported by Devilee et al. [15]. Allelic deletion rates in the 13q14 region varied from 27% of informative cases using the pG14E0.9 probe to 38% of informative cases using the p68RS2.0, the latter probe to the *RB* gene. Allelic deletion rates in a more distal region, 13q22–q34, were lower, 11–23% of informative cases, indicating specificity in the region of allelic deletion on chromosome 13. Similar results were reported by Sato et al. [18], where 21% of informative breast tumors exhibited a 13q allelic deletion.

The retinoblastoma (*RB*) gene stands as the candidate suppressor gene in this region. Discovered in retinoblastoma patients, a recessive mode of inactivation was demonstrated [19]. Transfection of *RB* has been reported in human retinoblastoma and osteosarcoma cells [20]. Significant changes in the *RB*-transfected cells were observed in culture morphology, colony formation in soft agar, growth rate, and tumorigenicity in mice, confirming *RB* as a cancer suppressor gene.

In breast tumors, a complicated pattern of *RB* DNA alterations and protein expression exists. Structural abnormalities to the *RB* locus was observed in 19% of informative cases by Varley et al. [21]. The same alteration was detected in metastases of these primary tumors, where available. No alteration at the *RB* locus were found in benign breast lesions. RB protein expression in the infiltrating ductal carcinomas was quantitated by immunoperoxidase staining. Of those cases exhibiting a *RB* deletion, a proportion of tumor cells were negative for RB protein. However, considerable heterogeneity existed in the percentage of tumor cells that were $RB^-$, from <5% to >95% of the tumor. While heterogeneity in tumor *RB* deletion among tumor cells in a specimen could account for a percentage of tumor cells staining with RB antibody, the data indicate that in some circumstances the remaining *RB* allele functions to maintain RB protein levels at an apparently normal level. Loss of RB protein expression was also observed in the absence of gene deletion or rearrangement.

Bookstein et al. [22] mapped the location of an *RB* allelic deletion in the

human breast carcinoma cell line MDA-MB-468. A 200-kb deletion was identified, largely confined to the RB1 gene. The data provide evidence for the specificity of *RB* allelic deletion in breast cancer.

### *2.5. Chromosome 16*

Allelic deletion on 16q was noted in 47% of informative breast cancers by Sato et al. [18] using the genomic probe p79-2-23. Of patients informative for 16q, 8 of 12 with allelic deletion exhibited lymph node metastases, while 8 of 20 patients without allelic deletions exhibited lymph node metastases ($p = 0.13$). Extensive mapping of multiple loci on chromosome 16 has not been reported to identify the region with the highest deletion rate, nor have specific putative suppressor genes been identified.

### *2.6. Chromosome 17p*

Multiple studies have demonstrated the allelic deletion of chromosome 17p loci in breast cancer (Table 1). Allelic deletion was consistently observed at a high rate at the YNZ22 locus, at 17p13.3. This probe has been thought to detect deletions relevant to the *p53* gene, which maps at 17p13.1. The actual *p53* probe is informative (heterozygous) in only a small percentage of cases, and allelic deletion data at the actual suppressor gene locus are therefore more difficult to obtain.

The *p53* suppressor gene is the most thoroughly studied cancer suppressor gene. Originally identified as an oncogene when mutated, the wild-type gene was demonstrated in murine cells [27] and human colorectal carcinoma cells [6] to be a cancer suppressor gene in transfection experiments. The major phenotypic effect in colorectal carcinoma cells is the virtual abolition of cell division [6]. Recessive inactivation of *p53* has been observed in colorectal carcinoma by allelic deletion and mutation of the remaining allele, and

*Table 1.* Somatic allelic deletion data on chromosome 17 in breast cancer

| Locus and/or probe | Location | Percentage of informative cases exhibiting deletion[a] | | | | | |
|---|---|---|---|---|---|---|---|
| p144D6 | 17p13.3 | 38 | 68 | | | | |
| YNZ22 | 17p13.3 | 48 | 61 | 58 | 58 | 52 | 52 |
| C3068 | 17p13.2 | | | 33 | | | |
| MCT35.1 | 17p13.1 | | | 44 | | | 32 |
| p53 | 17p13.1 | | | 27 | | 0 | 32 |
| EW301 | 17p11.2 | | 33 | | | | |
| p17H8 | 17 centromere | 25 | 4 | | | | |
| pNM23-H1 | 17q21 | 64 | | | | | |
| p2FC.6 | 17q21.3–q22 | | 22 | | | | |
| pTHH59 | 17q23–q25 | 29 | | 34 | | | |
| pRMU3 | 17q23–q25 | 38 | | | | | 18 |
| Reference | | 7, 23 | 15 | 24 | 25 | 26 | |

[a] Each vertical line of data summarizes the results of one cohort.

mutations in many cancer types have been found to cluster to several highly evolutionary conserved exons [28]. Mutated p53 proteins have been reported to have a longer half-life than wild-type proteins, resulting in an accumulation of p53 in some tumor cells [29].

Two recent studies have suggested that allelic deletion at YNZ22 may signal the presence of a second 17p suppressor gene, located more distal to *p53*. Coles et al. [24] reported no correlation of allelic deletion at YNZ22 (17p13.3) and *p53* (17p13.1). Allelic loss at the YNZ22 locus, however, was correlated with overexpression of p53 protein, suggesting that the putative suppressor gene at this locus acted to regulate *p53* expression. Chen et al. [26] reported that 52% of informative breast tumors were deleted at the YNZ22 locus, and noted a significant correlation between YNZ22 deletion and high proliferative capacity and DNA aneuploidy. Examinations of the same tumors for *p53* alterations produced contrasting results, with no evidence of deletion or rearrangement, and only 2 of 13 (15%) of the tumors exhibiting mutations in the conserved *p53* exons.

Leaving all YNZ22 data aside, Varley et al. [30] have reported the incidence of *p53* mutation in breast carcinoma using two techniques, sequencing of conserved exons and immunohistochemical staining using antibodies to the wild-type and putative mutated proteins. By immunohistochemistry 46 of 73 tumors (63%) stained positively with antibody to the mutated p53. In three carcinomas, sequencing of the conserved exons revealed mutations and confirmed the immunohistochemical analysis. Seven carcinomas were negative for p53 mutations in the conserved region. It is not apparent in the report whether these tumors were negative or positive for staining with the antibody to mutated p53. No significant staining was observed with either p53 antibody in normal ductal epithelium. Two of four cases in which both intraductal and infiltrating ductal carcinoma were evident in the same section stained positively for p53 protein in both components. In addition, 1 of 3 purely intraductal carcinoma cases stained positively for p53 protein. The data indicate that elevated p53 expression can be an early event in breast carcinoma. Additional prognostic studies have also reported a significant association between *p53* protein accumulation and markers of poor prognosis [45].

The *p53* suppressor gene has also been implicated in an inherited cancer syndrome, Li Fraumeni. A germ-line mutation of *p53*, in a region known to be a target of mutations in sporadic cancers, was reported in family members with this syndrome [31].

### *2.7. Chromosome 17q*

Two studies have indicated allelic deletion at 17q loci in breast cancer (Table 1). Cropp et al. [7] noted a 29–38% allelic deletion rate at 17q23–q25. These data were confirmed by Borrensen et al. [32], who found a 34% allelic deletion incidence at 17q23–q25. In the same cohort used by Cropp

et al. [7], Leone et al. [23] observed the allelic deletion of the *nm23-H1* probe, at 17q21, in 64% of informative cases.

*Nm23-H1* is a potential target of the 17q allelic deletion in breast cancer. *Nm23* was discovered on the basis of its reduced expression in murine melanomas of high vs. low metastatic potential. Allelic deletion of *nm23-H1* has been observed, in addition to breast cancer, in human non-small-cell lung, renal, and colorectal carcinomas; homozygous deletion of *nm23-H1* was observed in two colorectal carcinomas [23]. Transfection of the murine *nm23-1* cDNA into murine K-1735 TK melanoma cells resulted in a reduced incidence of primary tumor formation, significant reductions in tumor metastatic potential, and altered responsiveness to the cytokine TGF-beta (TGF-B) in vitro [33]. In breast carcinoma, reduced *nm23* RNA levels have been correlated with the presence of lymph node metastases [34] and significantly reduced patient disease-free and overall survival [35]. Whether the reduced expression of *nm23* is a consequence of its allelic deletion, mutation, or other regulatory interactions has not been established.

A chromosomal locus for early-onset, familial breast cancer was also mapped to chromosome 17q21 [36]. This locus has been reported to be distinct from *nm23-H1*, and may also be a target for 17q allelic deletion.

The human prohibition gene has also been localized to 17q21, and has antiproliferation activity. Mutations in two exons of the prohibition gene were identified in 4/23 breast cancers examined [46].

### *2.8. Chromosome 18*

Allelic deletion of chromosome 18q loci was mapped by Cropp et al. [7], and the highest frequency of allelic deletion was identified in the 18q21.3–23 region. The candidate suppressor gene in this region is in the colorectal cancer (*DCC*) gene, shown to be frequently altered in colorectal carcinoma [37]. Using probes to the D18S8 locus, located within the *DCC* gene, Devilee et al. [38] identified allelic deletion in 17% of informative cases and allelic gain (amplification) in another 10% of informative cases. Analysis of five loci for amplification on chromosome 18 indicated that a specific region was amplified, and no evidence for trisomy of chromosome 18 was found. The *DCC* gene has not yet been demonstrated to have suppressor function by transfection experiments, but if so the data indicate that multiple types of genetic alterations, not just recessive inactivation, may occur.

## 3. Suppressor genes for the metastatic phenotype

Tumor metastasis is a complex process requiring tumor cell invasion, movement, arrest in the circulatory system, angiogenesis, and colonization of a distant site. For many cancer types, including breast cancer, metastasis remains the major cause of patient death, and the regulation of its many

component processes is therefore of vital importance. Suppressor genes for tumor metastasis have not been defined by allelic deletion and mutation data in most cases. Often, a correlation was observed between quantitative expression of a certain protein, or mRNA, with metastatic potential. A suppressor function was confirmed by transfection of sense or antisense constructs into tumor cells. Despite the availability of in vitro assays for certain aspects of the tumor metastatic process (i.e., invasion of tumor cells through basement membranes), the only universally accepted measure of metastatic potential is the injection of cells into rodents, into either a tissue or the bloodstream, and quantitation of metastases formed. Four dissimilar metastasis suppressor genes have been reported in transfection experiments. None of the transfection experiments used breast carcinoma cells as targets; however, the genes may be widely applicable to the metastatic potential of many cancer cell types.

The first reported metastasis suppressor gene is Adenovirus 2 *E1a* [39]. Pozzatti et al. reported that transfection of rat embryo fibroblasts with activated *ras* induced both tumorigenicity and metastatic potential; however, cotransfection of *ras* and *E1a* resulted in tumorigenic, but poorly metastatic or nonmetastatic cells. Although E1a is not a normal cellular protein, it has been proposed that the viral genes mimic an endogenous, as yet undiscovered cellular gene relevant to cancer. *Ras* and *E1a* cotransfected rat embryo fibroblasts produced less type IV collagenase and expressed greater *nm23* RNA levels, suggesting that the metastatic phenotype may be altered by the coordinate regulation of a set of specific downstream genes.

A second metastasis suppressor gene has been reported in the same model system. Gattoni-Celli et al. [40] reported that *ras* and *E1a* cotransfected rat embryo fibroblasts maintained their low metastatic behavior in triple deficient mutant mice, eliminating the possibilities that T-lymphocytes, activated B-lymphocytes, or natural killer cells were responsible for its apparent low metastatic phenotype. However, the *ras* and *E1a* cotransfected cells expressed increased levels of major histocompatibility complex (MHC) class I genes, previously thought to be involved in immune recognition. In order to test the possibility that class I MHC genes may function in non-immunological mechanisms, the $H\text{-}2K^b$ gene was transfected into *ras*-transformed rat embryo fibroblasts [41]. Tumors formed by subcutaneous injection of the transfected cells into triple-deficient mice grew more slowly and exhibited decreased metastatic potential as compared to control cells. The data suggest the involvement of $H\text{-}2K^b$ in arms of the immune response, such as macrophage-induced cytotoxicity, or in suppression of nonimmunological aspects of the tumor metastatic process, possibly cell-cell adhesion.

In addition to regulatory genes, such as *E1a*, metastasis suppressor genes may also include genes that function to block an important effector mechanism in metastasis. An example of the latter case is the tissue inhibitor of metalloproteinase-1 (TIMP-1), which inhibits the activity of a broad

spectrum of activated collagenases used in tumor cell invasion. Transfection of antisense TIMP constructs into Swiss 3T3 cells was reported to reduce TIMP activity by 47–68%, indicating only a partial suppression of TIMP expression. The TIMP-antisense transfected lines were more invasive in in vitro amnion assays and exhibited greater tumorigenicity and metastatic capability in vivo [42].

A murine cDNA clone encoding *nm23*, discussed above, was demonstrated to significantly reduce the tumor metastatic potential of murine K-1735 TK melanoma cells upon transfection [33]. In unpublished experiments, *nm23-1* transfected K-1735 TK melanoma cells produced equivalent amounts of type IV collagenase, adhered to extracellular matrix proteins, and migrated in response to autocrine motility factor (AMF) to an extent comparable to control transfectants. The *nm23-1* transfected TK cells produced less of the protease stromelysin in vitro than the control transfectants, suggesting a potential mechanism for reduced invasiveness.

## 4. Summary

It is apparent that multiple genetic events occur in the development and progression of breast cancer. From the limited data available, no consistent temporal pattern of mutational events is required. This conclusion is consistent with data in colorectal carcinoma [43], where the number of mutational events, and not the order, appears to be relevant.

Several authors have questioned whether the multiple mutational events occur independently or whether significant associations were evident. Cropp et al. [7] postulated that two sets of mutational events occurred simultaneously in a higher degree of breast tumors than expected based on chance: Set 1 consisted of deletions on 11p, 17p, 18q, and *int-2* and *myc* amplifications; set 2 consisted of 17q, 1p, and 3p deletions. Sato et al. [18], analyzing another tumor cohort for simultaneous mutations, noted a correlation of 17p and 16q deletions, 13q and 17p deletions, and 17p deletion with *erb*B-2 amplification. Clearly, concordant data on this issue will require the use of large breast tumor cohorts for a comprehensive set of probes. The reasons why mutations to specific genes on different chromosomes tend to occur coordinately is unknown, but may involve common flanking and/or intron sequences at high risk for certain types of mutational events.

Another interesting question is the degree to which alterations, but not homozygous inactivation, of suppressor genes occur and its phenotypic consequences. In this chapter, evidence was presented for the amplification of a *DCC* allele in breast cancer and for variable RB protein expression in breast tumors as a consequence of allelic deletion. For many of the metastasis suppressor genes, a simple reduction in their expression, or an alteration in their expression over the normal cellular regulatory controls,

may be sufficient to fuel the metastatic process. The data suggest a more complex regulation of the cancer phenotype by suppressor genes than by recessive inactivation alone.

Why do many sporadic cancers, including breast cancer, appear to require alterations to multiple suppressor genes, as compared to diseases such as retinoblastoma, where a single suppressor gene appears to control the cancer phenotype? The answer to this question is unknown, but most theories are based on the hypothesis that suppressor genes act to control cellular responses to either other cells or signals in the microenvironment. In retinoblastoma all cells can carry a germ-line mutation. Cells carrying the *RB* mutation can interact with both the embryonic and differentiated microenvironments; the specific interaction of mutated cells with the embryonic retinal microenvironment may trigger the onset of retinoblastoma. In sporadic cancers, however, cancer cells expressing the altered suppressor gene interact only with the differentiated microenvironment. Furthermore, cancer cells with a suppressor gene alteration often comprise a nest of cells that interact with neighboring wild-type cells. Both the wild-type cells and the differentiated microenvironment may serve to stabilize the 'normal' phenotype, thus requiring multiple alterations for the development of the cancer phenotype.

Another area of intense research interest concerns the biochemical mechanism of action of suppressor genes. For instance, wild-type p53 protein binds certain viral proteins, again thought to be markers for endogenous proteins [43]; *p53* has been reported to be extensively mutated [28], and the p53 protein exhibits altered binding patterns to viral proteins when mutated [29]. Mutant p53 protein complexes with the wild-type protein [43] and p53 functions as a transcriptional activator of gene expression [44]. It is not known whether any or all of these biochemical functions is responsible for its suppressive effects on colon carcinoma cell division. Site-directed mutagenesis experiments, in which a particular biochemical function is inactivated by mutation and the mutated gene is then transfected into cancer cells to observe any changes in the cancer phenotype, are expected to determine the relationship between the suppressor protein biochemical activities known to date and the cancer phenotype. These experiments are obviously powerful, but limited, since we probably do not know all of the biochemical activities of any suppressor protein.

The potential clinical application of suppressor genes to breast cancer is difficult. Gene-therapy experiments are hindered by the inactivation of retroviral vectors by serum components and the necessity for tissue-specific delivery to minimize side effects. Should the biochemical mechanisms of suppressor gene action be clarified, they can be used as a screening system for the identification of drugs and/or biological response modifiers that upregulate cellular expression of a suppressor gene or interfere with the action of mutated suppressor proteins.

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# 4. The role of the retinoblastoma gene in breast cancer development

Yuen Kai Fung and Anne T'Ang

## 1. Introduction

Cancer is a multistep process involving the accumulation of many genetic mutations. These mutations can be the activation of dominantly acting protooncogenes or the inactivation of growth suppressor genes. The initial evidence for a role of growth suppressor genes in cancer development has come from somatic cell hybrid studies in which stable suppression of the tumorigenic phenotype of the hybrid was shown to correlate with the presence of specific chromosomes [1,2]. The observations that different tumor cells can complement each other in the suppression of the tumorigenic phenotype suggests that different chromosomes are involved in controlling tumorigenic expression in different cancer cells. These conclusions were confirmed by microcell fusion chromosome transfer techniques in which single chromosomes were shown to be able to suppress the tumorigenic phenotype of certain tumor cells [3]. An independent line of evidence for the role of recessive genetic mutations in tumor development has come from the study of familial pediatric tumors. It had been postulated that two mutational events are required for the development of retinoblastoma [4]. In hereditary retinoblastoma, since the germline mutation is present in all cells, a second somatic mutation in the target retinoblast is sufficient to allow for tumor development. Initial cytogenetic data and subsequent restriction fragment length polymorphism (RFLP) studies have provided more precise evidence for the involvement of chromosomal regions 13q14 in the development of retinoblastoma [5]. The isolation of the retinoblastoma gene, RB1, by us and others, allowed a confirmation of the recessive nature of cancer at a single gene level [6,7]. In hereditary retinoblastoma, where the mutations were detected as a homozygous deletion of the RB1 locus, identical structural changes were readily detected in one of the RB1 alleles in the constitutional cells of the patients [7]. In contrast, in nonhereditary retinoblastoma mutations were found exclusively in the tumor and not in the constitutional cells. These data are consistent with the course of mutational events postulated in the two-hit hypothesis. More interestingly these studies imply that the rate-limiting steps in the development of cancer are the loss

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

of genetic material that has a tumor suppression function. This conclusion is perhaps not surprising. Each of the mutational events in the multistep process of tumorigenesis represents a step that a cell must undertake towards full malignant transformation. While the activation of a proto-oncogene requires only mutation of one of the two alleles of the gene in a somatic cell, both alleles of a growth suppressor gene must be affected for its complete inactivation. Thus, the rate-limiting steps in tumorigenesis are often the inactivating mutation of growth suppressor genes.

In breast tumors a large number of genetic mutations have been documented. This includes the activation of various protooncogenes, such as *c-myc*, *int-2*, and *HER-2/neu* [8–11]. Cytogenetics and restriction fragment length polymorphism studies have revealed mutations of several specific chromosomal regions in breast tumors. The frequently involved chromosomal regions include 1p13 [12], 1q [13,14], 3p [15,16], 11p15 [17,18], 13q [19–21], 17p [22,23], and 17q21 [24]. The observed loss of heterozygosity in these chromosomal regions is suggestive of the mutation of a growth suppressor gene at or in the vicinity of the chromosomal region analyzed. The role played by each of these putative growth suppressor genes in the development of breast carcinoma awaits their isolation. However, at least two growth suppressor genes, RB1 and P53, have been identified and shown to be mutated in primary breast carcinoma.

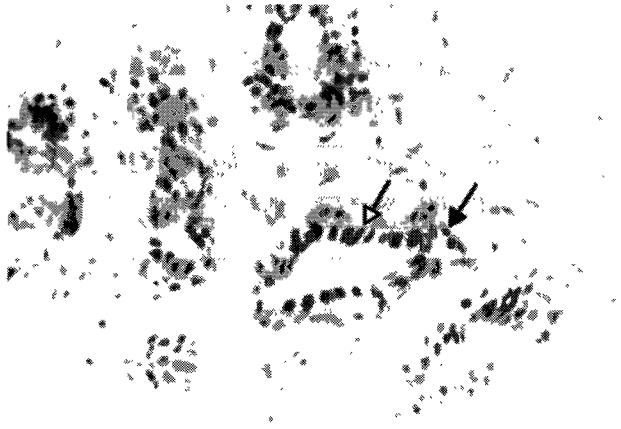
## 2. Inactivation of RB1 in breast cancer

It is well documented that surviving patients with hereditary childhood solid tumors have increased risk for other specific types of malignancies. Patients with the hereditary form of retinoblastoma frequently develop other primary malignancies, such as fibrosarcoma and osteosarcoma. Reduction to homozygosity at the RB1 locus has been detected in about 25% of osteosarcomas from patients with no prior history of retinoblastoma predisposition. This suggests that the RB1 locus is the link for the frequent clinical association of these different tumors. However, the inactivation of RB1 has been found in an even wider range of tumors. The rearrangement of the RB1 locus has also been detected in a number of adult tumors, such as bladder carcinoma and small-cell lung carcinoma [25,26]. While breast tumors have seldom been associated with retinoblastoma, a loss of heterozygosity on chromosome 13q has previously been reported in human ductal breast tumors [19]. Southern blot analysis of a number of primary tumors, as well as breast tumor cell lines, have revealed structural aberrations of the RB1 locus in about 25% of the breast tumor cell lines and in 7% of primary tumors [21]. However, no structural rearrangement in the constitutional cells of patients with hereditary breast tumors was revealed. This indicates that the RB1 locus may not be a hereditary component for the development of breast carcinoma.

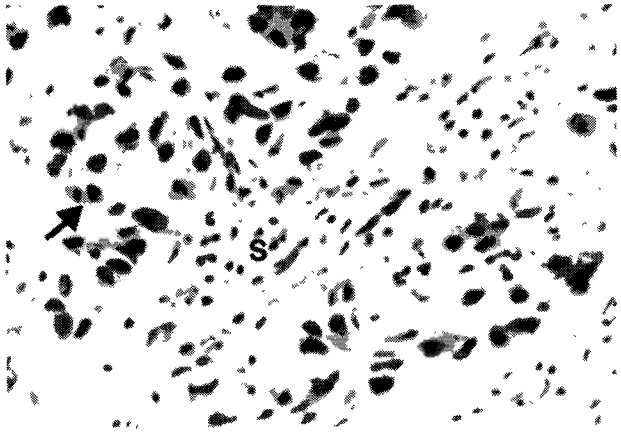
It should to be noted that while a relatively small number of steps are required for retinoblastoma tumorigenesis, the development of breast cancer involves many more rate-limiting steps. In fact, the time of onset of breast tumors is radically different from that of retinoblastoma. As stated before, RFLP studies of breast carcinoma have revealed the reduction to homozygosity on many different chromosomes, suggesting that there are a number of growth suppressor genes that are inactivated in the development of the disease. The inactivation of the retinoblastoma gene in breast carcinoma represents only one of the many different changes that are required for tumorigenesis. Predisposition to the development of breast tumor due to the inheritance of a defective allele of RB1, therefore, may not be unmasked. The question, becomes how the inactivation of the retinoblastoma gene contribute to the development of breast tumors.

### 3. The role of RB1 in breast carcinoma development

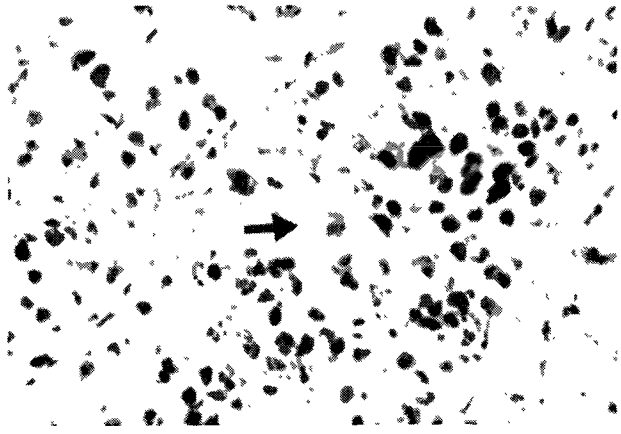
Although the function of the Rb protein is still unknown, some insight into the role of its inactivation in tumor development was obtained from immunohistochemical analysis of uncultured breast tumor tissues. The problems with analyzing the DNA from uncultured tumor samples are manyfold. A common problem is the heterogeneity of the tissue sample, since normal cells in an uncultured tumor sample could contribute to the hybridization signal. Residual hybridization signals detected in primary tumor DNA could be due to the presence of normal stromal tissues or of a subpopulation of tumor cells still expressing RB1. To circumvent this problem, we examined uncultured primary tumor samples by immunohistochemical staining using an antibody to the Rb protein so that its expression in tumor tissue sections can be analyzed in individual cells [27]. As shown in Figure 1A, the Rb protein is expressed in normal breast lobule epithelium. However, 16 out of 56 breast carcinoma samples analyzed showed the presence of unstained tumor cells in the tumor tissue sections (Figure 1B and 1C). Positive staining for the Rb protein was detected in 100% of the tumor cells from almost all the tissue sections of low-grade, well-differentiated tumors. Ductal structures were readily detected in these tumor sections (Figure 1B). However, tissue sections from poorly differentiated tumors revealed the presence of tumor cells unable to express the Rb protein (unstainable by the Rb antibody) (Figure 1C). Morphologically tumor cells stained positive with the antibody to the Rb protein could not be distinguished from those that were stained negative. The proportion of tumor cells that had lost Rb protein expression in this group of tumors ranged from below 5% of the tumor cells to around 95% of cells. Cells that did not stain were distributed either in clusters or scattered throughout the tumor (Figure 1C). In five cases in which there were no detectable alterations to RB1 by DNA analysis, unstained tumor cells within the sample



A




B



C

were still detected. Altogether approximately 29% of the breast carcinoma samples examined had various degrees of loss of RB1 protein expression.

Since tumor cells that were stained negatively appear in clusters among positively staining tumor cells, the loss of the RB1 expression must be an event that happened after tumor formation. The inactivation of RB1 is therefore not an initiation event for breast carcinoma; rather, it is a late event associated with progression of the tumor to a more malignant stage. Further support for this conclusion comes from correlating the alteration of RB1 to the histopathological grade or to the spread of the tumor (Table 1). The percentage of tumors with loss of Rb protein expression increases with the grade of the tumor (defined as the state of differentiation of the tumor). In general, the frequency of genetic changes in RB1 increases with poorer differentiation of the tumor, indicating that the inactivation of RB1 correlates with the loss of the ability of the cells to differentiate. A role of RB1 in the control of differentiation perhaps is not unexpected. In retinoblastoma, tumors occur typically in children under the age of seven (and mostly under the age of four), by which time the retinoblasts would have completed their differentiation. The subsequent inactivation of the RB1 locus would have no effect on the growth of the cells. This suggests that the normal function of the Rb protein is one of controlling differentiation. The inactivation of RB1 may contribute to the progression of breast tumors by rendering the cells incapable of differentiation.

When the frequency of loss of RB1 expression was compared to the stage of the tumor, a clear correlation was observed (Table 1). The percentage of tumors with tumor cells that had lost the expression of RB1 increased dramatically with the more advanced stages of the disease. About 50–80% of tumors that exhibited local invasion or distant metastases showed the loss of RB1 expression. Therefore, in addition to losing the ability to differentiate, the inactivation of RB1 expression in tumor cells also correlates with the gain in its ability to grow in a foreign environment.

## 4. Suppression of cell growth in vitro

If the inactivation of RB1 contributes to the late-stage progression of the tumor, what effect would it have if the defective RB1 is replaced with a wild-type cDNA of the Rb gene? One could predict that tumor progression

*Figure 1.* Immunohistochemical detection of RB1 protein in human breast tissue. A: Normal breast lobule with nuclear and, to a lesser extent, cytoplasmic staining of epithelium (open arrow). Myoepithelial cells appear negative (closed arrow). B: Typical staining pattern of those carcinomas in which essentially all tumor cell nuclei react (arrow) with minor variation in staining intensity. Surrounding stromal cells (s) show no evidence of reactivity with the antibody. C: A small group of tumor cells stained positive (arrow), whilst the rest of the tumor cells are negative. This was the major area of staining within the carcinoma. All magnifications ×250.

*Table 1.* Distribution of the frequency of loss of expression compared to histopathological grade or to spread of the disease

| Category | Percentage of tumors with loss of expression |
|---|---|
| Grade[a] | |
| I | 0 (n = 5) |
| II | 19 (n = 21) |
| III | 48 (n = 23) |
| Stage[b] | |
| 1 | 11 (n = 23) |
| 2 | 46 (n = 13) |
| 3 | 80 (n = 5) |
| 4 | 50 (n = 2) |

[a] Grade I tumors are the most, and grade III the least, well differentiated tumors.
[b] Stage 1 is a tumor only, stage 2 is a tumor plus involvement of local lymph nodes, stage 3 is a large tumor with skin tethering (local invasions), and stage 4 is a tumor plus distant metastases.

would be inhibited and that the cells would not be able to grow in a foreign environment (to metastasize). To address this issue, we replaced the defective RB1 gene in the breast tumor cell line MDAMB 468 with a wild-type cDNA of RB. MDAMB468 is a breast tumor cell line from an infiltrating ductal breast carcinoma that has previously been shown to contain a homozygous deletion of the entire RB1 locus [20,21]. For these studies, the wild-type RB1 cDNA was cloned into several different types of vectors, each containing a different promoter. The promoters used were the human beta-actin promoter, the SV40 promoter, and the MuLVLTR. Cells were transfected with each of these constructs, and the growth of the transfected cells was followed by immunostaining at different days after transfection. Figure 2 shows the transfection of MDAMB468 with either SVRB1-neo or with a control plasmid pK1-neo. Both plasmids were under the control of the SV40 promoter. The plasmid pK1-neo contains a mutant SV40 large T-antigen gene that is defective in transformation. Since the cells do not normally express the mutant SV40 large T-antigen, pK1-neo was used as a control because it afforded a very clean background for immunostaining. The transfection efficiency of SVRB1-neo and the control plasmid pK1-neo measured at day 3 were similar; both were at an efficiency of $10^{-3}$. After selection for 2 weeks in G418, differences in the proliferative capacity of the transfected cells became apparent. At day 12, cells transfected with pK1-neo divided to form colonies of several hundred cells. However, those transfected with SVRB1-neo were severely growth retarded and remained as single cells rather than forming colonies. This difference in the cell growth rate was even more pronounced at day 24, by which time cells transfected with pK1-neo had expanded into colonies consisting of more than $10^3$ cells at an efficiency of $10^{-3}$ to $10^{-4}$. Each of these colonies were stained positively

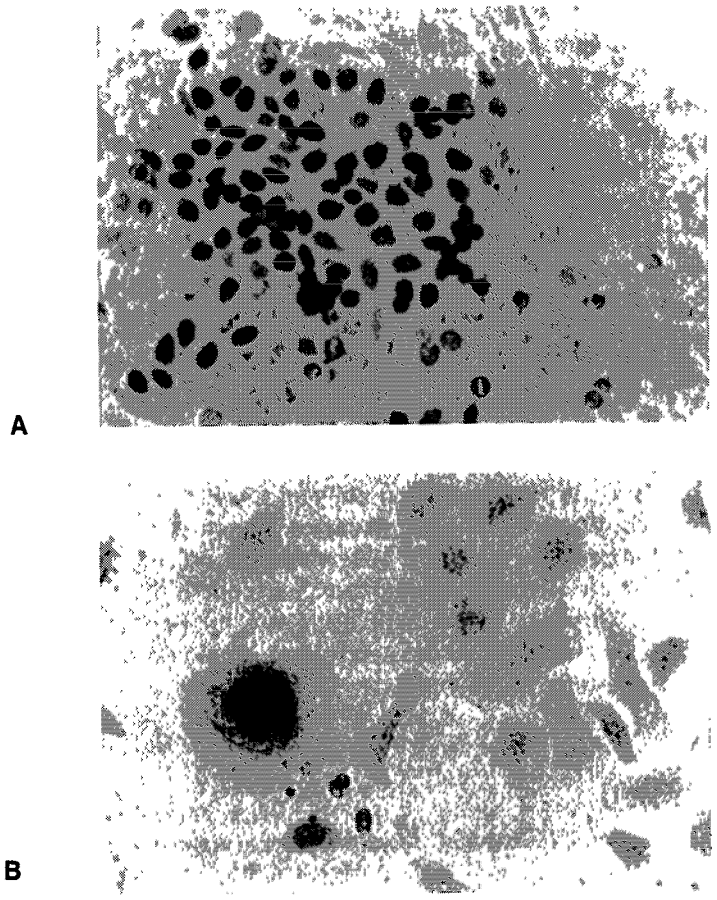



*Figure 2.* In vitro growth behavior of MDAMB 468 transfected with (A) PK1-neo and (B) SVRb1-neo at day 24 after transfection. $10^7$ exponentially growing MDAMB 468 were transfected with 100 µg of either PK1-neo (A) or SVRb1-neo (B). The transfected cells were plated, but in RPMI 1640 + 10% FCS at a density of $2 \times 10^4/cm^2$ in 100-mm dishes for 24 hours. The medium was then changed to one containing 100 µg/ml G418 (GIBCO). At days 3, 12, and 24, one dish each was taken for immunostaining with (A) PAb100 or with (B) RB1-Ab B or RB1-Ab20.

with PAb 100, an antibody to SV40 large T (Figure 2A). In contrast, all the cells transfected with the SVRB1-neo plasmid and that stained positively for the Rb protein were severely growth retarded and greatly enlarged in size (Figure 2B). Almost all of these cells that were reproducibly obtained at an efficiency of $10^{-3}$ to $10^{-4}$ appear as single undivided cells. All the multicell colonies adjacent to these single RB positive cells were stained negatively with the RB antibody. Moreover, none of these collonies (30/30) isolated contain immunoprecipitable Rb protein. While cells stained positively for Rb expression at day 3 were of the same size and morphology as the

surrounding cells, they have become flattened and greatly enlarged by day 24. Many of these giant cells also contained multiple nuclei or vacuoles. Evidence for cessation of DNA synthesis in these giant cells came from the absence of observed staining when cells were labeled briefly with bromodeoxyuridine (BrdU) and were immunostained with antibody against BrdU. These cells, therefore, resemble end-stage senescent cells. These data suggest that the replacement of the Rb protein in these tumor cells prevented their growth in vitro.

The RB1 gene has previously been reported to specifically suppress the tumorigenic phenotype of certain tumors [28,29] but has no effect on their growth in vitro. Our data on breast tumors showed that the RB1 cDNA prevent the growth of these tumors in vitro. It is possible that different tumors respond differently to RB1. We observed, however, that in many other tumor cell lines, such as the osteosarcoma cell line Saos-2 and the fibrosarcoma cell line HT1080, cells that were not growth inhibited by a transfected RB cDNA in vitro were also not suppressed in vivo. The in vitro growth suppression function of RB1, therefore, could not be dissociated from its in vivo suppression function. Why different cell lines appeared to respond differently to RB1 remains to be determined. Nonetheless, the ability of RB1 to suppress the growth of breast tumor cells in vitro is consistent with the conclusion that its inactivation promoted the progression of the disease by the removal of a growth constraint.

## 5. Summary

The observation that the human retinoblastoma gene is inactivated in about 20% of breast carcinomas indicates that it may be important in the development of these tumors. The fact that the loss of RB1 expression correlates with the progression of the disease, and especially with the inability of the cells to differentiate, is consistent with the clinical observation that retinoblastoma does not occur in children in whom the target cells have already fully differentiated. This suggests that the normal function of RB1 is to promote differentiation. It is possible that the loss of the ability of a cell to differentiate contributes to its ability to grow in a foreign environment (metastasis), but this hypothesis remains to be tested. Our observation that the overexpression of RB1 suppresses the growth of these tumor cells in vitro is consistent with this hypotheses.

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# 5. Mammary-Derived Growth Inhibitor (MDGI)

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## 1. Introduction

In contrast to the steadily increasing number of growth factors capable of stimulating cells to synthesize DNA and to divide, very little is known about their potential antagonists, which are supposed to contribute to balanced growth and development by inhibiting cellular proliferation.

The present volume describes in detail the hitherto best-investigated growth inhibitors for mammary epithelial cells, the family of beta-type transforming growth factors, and the recently discovered mammostatin. Actually, along with MDGI, they are the only known growth inhibitors produced by mammary epithelial cells. A critical evaluation of growth inhibitor research that applies the structural and functional criteria of the biology of growth-stimulating factors will result in the conclusion that we are still seeking the hormones transducing inhibitory signals for cessation of growth.

The proof for the existence of growth inhibitors acting in an autocrine or paracrine pathway in normal mammary gland physiology would help to explain how this periodically regenerating epithelium proceeds through different cycles of ductal growth and lobuloalveolar differentiation. Also, loss of corresponding signals may be related to the mechanism of escape of transformed cells from normal control.

What is the evidence for the participation of growth inhibitors in the normal development of the mammary gland? In fact, ductal growth, morphogenesis, and functional differentiation can be driven in vitro by a combination of steroid hormones and prolactin [for a review, see 1]. There are no growth factors, neither stimulatory nor inhibitory, to be added to achieve glandular development. On the other hand, ablation of endocrine glands causes the inhibition of mammary epithelial cell growth in vivo. These findings lead to the question of whether locally acting factors, the synthesis of which is under hormonal control, could be involved. It is our interest to search for those peptides, in particular, growth inhibitors.

In this chapter shall be discussed the properties of a 14.5-kDa polypeptide that we believe is involved in the local control of proliferation and differ-

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

entiation of the mammary gland. The purification and in vitro proliferation assays, cDNA cloning, and in vivo expression in the bovine mammary gland have been described in detail elsewhere [2–4].

Therefore those points will be touched on only in brief before we discuss functionally interesting sequence homologies, the growth-modulating activities of synthetic MDGI peptides, and hormone-dependent MDGI expression in mouse mammary gland explant cultures.

## 2. Purification

The initial description of growth-inhibitory activities derived from the mammary tissue dates back to 1977, when W. Lehmann, P. Langen, and coworkers began to characterize the effects of extracts obtained from ascites fluid of Ehrlich mammary carcinoma cells on the growth of these cells [5,6]. The assay system had originally been developed as a screening test for antineoplastic agents. Ehrlich cells, harvested from the peritoneal cavity, are cultured in the presence of test samples for 24 hours and growth is judged by the estimation of cell number [7].

Since a successful isolation of the inhibitory principle is obviously required, the availability of large amounts of starting material, bovine mammary gland tissue, was considered as a source. The 'chalone' concept [8], claiming the existence of tissue-specific autocrine growth inhibitors, and the fact that Ehrlich ascites tumor had once been derived from a murine mammary carcinoma [9], guided these studies. Indeed, extracts from lactating bovine mammary tissue displayed similar inhibitory activities as the material previously obtained from ascites and Ehrlich cells [10].

Various purification schemes employing common chromatographic and electrophoretic techniques [for a review and details of recent methods see 3] revealed that the majority of inhibitory activity obtained from lactating bovine mammary tissue is associated with a 13- to 15-kDa protein [11]. Evidence for the identity of this protein with the inhibitory principle provided immunoneutralization experiments [12].

The putative growth inhibitor was purified to homogeneity and the amino sequence of the protein designated as mammary-derived growth inhibitor (MDGI) was determined [13].

## 3. Structural features and functional implications

The amino acid sequence of the 14.5-kDa MDGI (Figure 1A) revealed no homology to any of the hitherto known growth inhibitors, such as transforming growth factor-beta or the interferons [13]. A striking homology was, however, evident to members of a large family of hydrophobic ligand binding proteins comprising fatty acid-binding proteins (FABP) [14,15],

cellular retinoid binding proteins, myelin P2, and others (Figure 1). For the adipocyte, liver, and intestinal FABP, a relationship between their expression and the differentiated stage of the tissue has been documented [15], a property also discussed before for cellular retinoid binding proteins [16]. The closest structural relationship was found to fatty-acid binding protein(s) of the cardiac type. Indeed, the amino acid sequence of bovine cardiac FABP predicted from a cDNA sequence [17] was found to be 95% homologous to the amino acid sequence of bovine MDGI. Amino acid sequencing of MDGI revealed a number of 'microheterogeneities,' i.e., in eight cases [13] (R. Kraft, personal communication] two residues were found in comparable amounts in the same position of the sequence [13]. This finding suggests that the material used for sequencing was not entirely homogeneous and contained at least two slightly different 'isoforms' of MDGI, which could both have contributed to the biological activity of MDGI (MDGI A and MDGI B, Figure 1). One of them is identical to bovine heart FABP.

Bovine MDGI and homologs

```
          1        10        20        30        40        50
          |        |        *|        |        |         |
MDGI A    VDAFVGTWKLVSSENFDDYMKSLGVGFATRQVGNMA--KPTLIISVNGDTVI
MDGI B    ...........D.K.....................T--...T..E.......
(H-FABP)
MP2       SNK.L............E...A....L...KL..L.--..RV...KK..IIT
CRABP      PN.A....MR......ELL.A...NAML.K.AVA.AS..HVE.RQD..QFY

                   60        70        80        90           100
                    |         |         |         |            |
MDGI A    IKTQSTFKNTEISFKLGVEFDETTADDRKVKSIVNLDEGKLVQVQK----WNGQ
MDGI B    ..................................T..G....H...----....
(H-FABP)
MP2       .R.E.P...........Q..E.....N..T..T...AR.S.N....----...N
CRABP     ...ST.VRT...N..V.EG.E.E.V.G..CR.LPTWENENKIHCTQTLLEGD.P


                  110       120       130
                    |         |         |
MDGI A    ETSLVREMVDGKLILTLTHGTAVCTRVYEKQ
MDGI B    ..........................T....A
(H-FABP)
MP2       ..TIK.KL....MVVECKMKDV....I...V
CRABP     K.YWT..LANDE.I..FGADDVV...I.V.E
```

*Figure 1.* Amino acid sequence of MDGI and closely related bovine forms. A: The MDGI-A sequence was obtained by amino acid sequencing [13]. In ten positions MDGI-A differs from heart FABP [18], which is identical to the cDNA-deduced bovine MDGI-B sequence. In 7 of the 10 differences, two residues were found for MDGI-A [13]. Differences in positions 40 and 43, and the lack of C-terminal alanin in MDGI-A are unambigous. Only nonidentical residues are shown for the related proteins. H-FABP = heart fatty-acid binding protein [17]; MP2 = myelin P2 [72]; CRABP = cellular retinoic acid binding protein [73].

Screening of a cDNA library derived from bovine lactating mammary gland revealed only one MDGI form, which turned out to be identical with the cardiac sequence [18] (Figure 1, MDGI B).

Other MDGI species have not been found among nine cDNA clones from the bovine lactating mammary gland library. Furthermore, differential hybridization of PCR fragments amplified from bovine mammary cDNAs from two animals and from bovine genomic DNA with MDGI-specific primers did not give rise to another MDGI form [18]. These results suggest that the MDGI species identical with cardiac FABP is the abundant form in terminally differentiated mammary gland (MDGI-B, Figure 1), whereas the other isoform (MDGI-A, Figure 1) constitutes a minor component in this tissue.

It cannot be excluded that more than one gene for MDGI/cFABP does exist. Post-translational mechanisms are not sufficient to explain the eight amino acid differences between the bovine MDGI forms A and B.

One could assume that during the development of the mammary gland two different MDGI forms may become expressed, one of which is abundant during lactation and identical with the cardiac FABP, whereas the other one, is produced at an earlier stage of development. The hypothesis is supported by two lines of experiments.

First, an MDGI form with a primary structure not identical with the cardiac FABP has been detected by screening of a cDNA library that was derived from the mammary gland of the pregnant mouse (B. Binas et al., in press). The cloned mouse MDGI cDNA predicts a protein sequence that differs in nine positions from that of the murine cardiac cDNA-derived sequence of FABP [19] (Figure 2). The 5′-untranslated regions of the two proteins are completely different.

Second, by protein fractionation of the mammary gland of pregnant cow, we have recently detected a slightly 'shortened' MDGI form (R. Brandt et al., manuscript in preparation). This protein comprises less than 1% of MDGI and is not present in the lactating gland. It is immunologically related to MDGI; however, it does not crossreact with an antibody directed to the C terminus of MDGI. The amino acid sequence has not yet been determined, but the available data suggest that this protein is processed from the minor MDGI-A form rather than from an abundant MDGI-B, which is supposed to be present in terminally differentiated tissue.

The homologies of the mouse MDGI form with the bovine MDGI and the bovine cFABP amount to 80% and 84%, respectively.

Three genes located on different chromosomes have been found with a probe for the cardiac FABP in mouse [20]. One of them might code for the MDGI transcript identified by us.

Two mammary-gland-derived FABPs have also been isolated from the rat [21]. One of the two forms that were isolated from lactating mammary glands were partially sequenced and turned out to be identical to the corresponding cardiac FABP sequence. Sequence data for the second, less

Murine MDGI and homologs

```
           1        10         20         30         40         50
           |        |          |          |          |          |
MDGI       ADPFVGTWKLVDSKNFDDYMKSLGVGFATRQVASMTKPTTIIEKNGDTIT
H-FABP     .ER.....................................................
ALBP       C.A........S.E.......EV.......K..G.A..NM..SV...LV.

                   60         70         80         90         100
                    |          |          |          |          |
MDGI       IKTQSTFKNTEINFQDLGIEFDEVTADDRRVKSLETLDGAKLIHVQKWNG
H-FABP     ..............-.............K....V....G........D.
ALBP       .RSE........S.K-..V....I.........II....GA.VQ....D.

                  110        120        130
                    |          |          |
MDGI       QEITLTRELVDGKLILTLTHGSVVSTRTYEKQA
H-FABP     ..T............................E.
ALBP       KS..IK.KRDGD..VVECVMKG.T...V..RA
```

*Figure 2.* Amino acid sequence of murine MDGI and closely related murine proteins. The MDGI sequence has been deduced from cDNA cloning. The sequence for heart FABP (H-FABP) and the adipocyte lipid binding protein (ALBP) were taken from Tweedie and Edwards [19] and Bernlohr et al. [22], respectively. MDGI differs in nine positions from the cDNA-derived H-FABP sequence.

abundant form are not available, but it might correspond to MDGI-A in cattle.

In summary, these results suggest the existence of at least two MDGI forms in the mammary glands of cattle, mouse, and rat. One of the forms is identical with cardiac FABP.

As mentioned before, for some types of FABPs the limited organ distribution and a development-dependent manner of topological tissue expression indicates some role for tissue-specific processes during differentiation [14,15]. Bovine cardiac-type related FABPs have been detected in the heart, mammary gland, skeletal muscle, and brain. Rodent cardiac-type FABP expression is restricted to the heart, mammary gland, adrenal gland, skeletal muscle, kidney, aorta, placenta, and testes. It would be of more general importance to elucidate the identity of the different cFABP/MDGI forms expressed in the different tissues.

The pattern of MDGI forms in vivo might be complicated further by post-translational modifications: A potential site for tyrosine phosphorylation exists at Tyr 19 (Figure 1). The sequence around this tyrosine is rather conservative and is highly homologous to the corresponding sequences of the adipocyte lipid-binding protein — p422, aP2, and ALBP [22] (Figure 1). For ALBP, phosphorylation of Tyr 19 by the insulin receptor tyrosine kinase has been shown in vitro and in vivo [23,24] and has been suggested to

be linked with insulin receptor signaling [23]. The phosphorylation in vitro was shown to be activated by the binding of fatty acids to ALBP [25]. Fatty-acid binding has been suggested to induce a conformational change of ALBP, leading to a facilitated access of the kinase to Tyr 19. Though this has not yet been demonstrated, it seems likely that MDGI can undergo phosphorylation at Tyr 19 and that phosphorylated and nonphosphorylated forms of MDGI may coexist in the cell. Second, the chemical analysis of MDGI preparations revealed the presence of nonextractable, i.e., probably covalently bound, fatty acids [2]. The sites and the exact nature of this possible modification are not yet known. Finally, as outlined later in more detail, MDGI, like its relatives, can bind long-chain fatty acids, creating a further level to post-translational modification by ligand binding. For all these possible modifications, as for the structural microheterogeneities discussed above, the functional significance remains to become elucidated.

The C-terminus of MDGI residues 126–130 are identical to residues 108–112 of bovine growth hormone [26] (Figure 1). This stretch of amino acids is part of a sequence of growth hormone that is essential for its biological activity [27]. The homology raised the question of whether MDGI or MDGI-derived peptides might compete with growth hormone for binding to the growth hormone or prolactin receptor. This seems, however, not to be the case, since MDGI-derived C-terminal peptides comprising the homologous sequence do not compete with growth hormone or prolactin for binding to their respective receptors (P.A. Kelly, personal communication). As we shall describe in Chapter 4, this does not exclude an indirect functional interaction between synthetic MDGI peptides and prolactin-dependent intracellular pathways.

The three-dimensional structure of MDGI is most likely very similar to that of the closely homologous proteins for which X-ray crystallography has been accomplished [28–30]. Those proteins have been shown to fold into a barrel-like structure, largely composed of beta-sheets enclosing a cavity that harbors the lipid ligand. The recently presented structure of bovine cardiac FABP [30] presents a fully applicable picture of one of the MDGI forms. Data obtained by analytical ultracentrifugation of MDGI [31] or by theoretical calculations based on atomic coordinates [32] are in accordance with the crystallographic data.

As in the case of the structural relatives of MDGI, the sequences of the cloned cDNA for bovine MDGI-B and murine MDGI lack a signal sequence for membrane translocation [18] (B. Binas et al., in press). This finding raises several questions with regard to the physiological significance of the observed biological effects of MDGI added extracellularly to target cells. If MDGI is supposed to act in an autocrine manner as a growth inhibitor, how can it reach the putative receptor on the target cells if it is not secreted? If most of MDGI has an intracellular localization, what is the function of this protein inside the cell?

With regard to secretion, an analogy might exist to other growth factors that also lack a signal sequence like FGF and PG-ECGF [33,34]. In those cases cell damage as a possible way of secretion, or the existence of related factors with a signal sequence as physiological ligands of the respective surface receptors, have been discussed. Both possibilities might also be considered for MDGI. It should be noted that the currently available data do not allow the exclusion of some 'leakage' of MDGI into the extracellular compartment. In cryosections of mouse mammary glands, MDGI-related immunostaining has been observed over the extracellular matrix (F. Vogel, unpublished observation). For rat cardiac FABP small amounts were found to be continuously present in serum with an increase in experimental ischemia [35]. Also, a more distant structural relative of MDGI — gastrotropin — [36] is supposed to be present and to act physiologically in the extracellular compartment. In addition, an immunologically crossreacting growth inhibitory protein — FGRs [37,38] — has been purified from conditioned media of 3T3 fibroblasts [37]. Alternatively, the physiological site of action of MDGI might be the cell interior. Whereas we have so far not been able to demonstrate a surface receptor for MDGI, experiments with $^{125}$I-labeled MDGI and Ehrlich cells revealed that at least a part of the protein is taken up by the cells and remains stable as a 14.5-kDa molecule over hours of incubation (Müller, Böhmer, Vogel et al., unpublished data).

With regard to the high amounts of MDGI in lactating tissue (in contrast to very small levels in all other situations, for example, target cells in vitro!), the key to understanding might be a dissection of the various possible forms (see above). Only a minor form of MDGI (MDGI-A?) might participate in regulation, whereas the bulk form(s) could serve a distinct function, possibly in fatty-acid mobilization and transport, as discussed for FABPs in recent reviews [4,14,15].

As expected from the structural homologies to lipid-binding proteins, MDGI was revealed to be a lipid-binding protein as well [39]. Among the various potential ligands tested, only long-chain fatty acids were found to become bound to MDGI with characteristics very similar to other fatty-acid binding proteins [40] (Table 1). Therefore MDGI, or rather its predominant form in the lactating mammary gland, could well be referred to also as 'bovine mammary fatty acid binding protein.'

An obvious interesting question is whether ligand binding could be related to the biological activity of MDGI. In principle, transport of a physiologically active (growth-inhibitory) ligand or depletion of a ligand essential for cell growth could be putative ways of MDGI-ligand interactions in cell growth inhibition.

As outlined in Table 1, so far the search for a biologically potent ligand has failed. In particular, no binding of any of the tested eicosanoids could be detected. In contrast, for rat liver FABP, binding of eicosanoids (15-HPETE, 5-HETE, 5-HPETE, $PGA_1$, $PGA_2$) with a relatively high affinity

*Table 1.* Lipid binding to MDGI

| Lipid | Binding assayed by | | Apparent dissociation constant ($10^{-6}$M) |
|---|---|---|---|
| | Direct binding of labeled ligand | Displacement of labeled palmitic acid | |
| Oleic acid | + | + | 0.9 |
| Arachidonic acid | + | + | 1.5 |
| Palmitic acid | + | + | 2.6 |
| Myristic acid | n.d. | + | n.d. |
| Caprylic acid | n.d. | − | |
| Retinoic acid | − | − | |
| Retinol | − | − | |
| Retinol acetate | n.d. | − | |
| Estradiol | n.d. | − | |
| Prostaglandin E1 | − | − | |
| Prostaglandin E2 | − | − | |
| Thromboxane B2 | n.d. | − | |
| Thromboxane A2 Agonist | n.d. | − | |
| 15S-HETE | − | − | |
| Tetraiodothyronine | − | n.d. | |
| (−)-isoprenaline | n.d. | − | |

ND = not determined.

has been demonstrated [41,42] and has been linked to a role of this protein in mitosis and as a target for carcinogens [43]. Long-chain fatty acids, however, added to cell cultures at concentrations as low as those of MDGI in the growth assay [11–13], fail to inhibit cellular proliferation. Also depletion of essential fatty acids by MDGI is unlikely to form the basis for the inhibitory effect, considering the relatively low affinity for fatty acid binding. In addition, direct uptake measurements revealed that long-chain fatty acids bound to MDGI are even more easily taken up by the target cells than those added in free form [39]. As outlined in detail below, we found that synthetic peptides derived from the MDGI sequence have similar biological activity as MDGI. These peptides are, however, unable to bind fatty acids [40]. We therefore conclude that the MDGI ligand-interaction is not directly involved in its biological activity.

It is likely that the affinity of MDGI to fatty acids is associated with the synthesis and secretion of lipids into the milk. Indeed MDGI is abundantly present in 'milk fat globules' [44] and might have some protective functions for the descendants. It remains a possibility that an indirect effect of ligand binding, in terms of a structural change of MDGI upon ligand binding, might be necessary for full functional activity. Evidence for such a structural change has been obtained by analytical ultracentrifugation studies [31]. An analogy can be drawn to the one suggested to form the basis of the faci-

*Table 2.* Inhibition of DNA synthesis by MDGI in normal and transformed cells: Restimulation test with serum-started cells. $^3$H-TdR pulse after 14 hours starvation

| | | % Inhibition |
|---|---|---|
| MATU | (human ductal carcinoma) | 40–50 |
| T 47 D | (human ductal carcinoma) | 40 |
| MCF-7 | (human ductal carcinoma) | 0–10 |
| mMa Ca | (mouse, adenocarcinoma) | 30 |
| Normal human mammary epithelial cells | | |
| 2nd passage | | 0 |
| 3rd passage | | 23 |
| 5th passage | | 30 |
| 6th passage | | 60 |
| 12th passage | | 100 |
| 14th passage | | 100 |
| NRK | (rat kidney fibroblasts) | 0 |
| A431 | (squamous epidermoid carcinoma) | 0 |
| P19 | (mouse embryonic stem cells) | 0 |

litated access of tyrosine kinase to Tyr 19 in ALBP upon ligand binding [25].

## 4. Biological activities

### *4.1. Growth inhibition of mammary epithelial cells*

Ehrlich cells from the stationary phase of growth in vivo were first used to assay growth-inhibitory activities in vitro [5]. As outlined in detail previously, in these cells half-maximal inhibition was obtained with about $10^{-10}$ M MDGI (1 ng/ml). Inhibition was abolished by simultaneously adding MDGI with EGF, insulin, or 2′-deoxycytidine [12,45]. Ehrlich cells taken from the logarithmic phase of in vivo growth do not respond to MDGI. This is in accordance with pulse cytophotometry measurements indicating that cells late in the $G_1$-phase were more sensitive towards MDGI action than S- or $G_2$-phase cells [7].

The response to MDGI of permanent mammary carcinoma cell lines, normal human mammary epithelial cells, and nonepithelial cells is compared in Table 2. The proliferation assay is based on serum starvation, followed by a restimulation period with fresh medium. MDGI was present during the restimulation period for 16–20 hours. Flow cytophotometric measurements proved them to be arrested in $G_1/G_o$, which was a prerequisite for growth inhibition. The data indicate that various mammary epithelial cells are sensitive to the growth-inhibitory action of MDGI. However, there is no

obvious relationship between sensitivity towards MDGI and growth parameters of human mammary carcinoma cells (such as their ability to clonogenic growth) or origin (murine or human). In a soft agar growth assay with MATU cells, treatment with MDGI for 6 days did not exceed growth inhibition obtained in monolayer culture (K. Eckert, unpublished data). The enhanced sensitivity of normal mammary epithelial cells towards MDGI [46] is interesting. While the inhibition of growth of Ehrlich cells could be prevented completely by low concentrations of fetal calf serum, insulin, or EGF [47], restimulation of normal mammary epithelial cells was performed in the presence of 10% fetal calf serum, EGF, insulin, and pituitary extract. The degree of inhibition increased with the passage number. This finding gives rise to speculations that MDGI might act on normal, rather than on transformed, cells and that in later passages cells may have lost their proliferative capacity, and/or some differentiated functions may have been selected in certain responsive cell clones.

### *4.2. Stimulation of differentiation*

As discussed before, in the mammary gland from pregnant mouse an MDGI form was detected that differs from murine-heart FABP. Expression studies clearly indicate a relationship between the onset of endogenous MDGI expression and differentiation of the mouse and bovine mammary gland (see Chapter 5). It, therefore, seems reasonable to assume that the protein may fulfill a function related to differentiation.

In order to directly prove a function of MDGI during differentiation, we established an organ explant culture system with mouse mammary glands (Binas et al., in press). Furthermore, we have taken advantage of a synthetic peptide comprising the last 11 C-terminal amino acids [121–131] (to prevent dimerization and subsequent loss of activity Cys was replaced by Ser) of MDGI. The peptide P108 behaves in several aspects as MDGI. It partially blocks insulin and 2′-deoxycytidine [2], and it reverses the supersensitivity of $beta_2$-adrenergic receptors towards isoprenaline (Chapter 4) [40].

If mammary explants from mid- to late-pregnant Balb c-mice were cultured for several days in a serum-free medium containing prolactin, insulin, and hydrocortisone, lobuloalveoli expressed the typical phenotype of a functionally differentiated mammary gland, i.e., the alveolar cells are vacuolized, secretory active, and they snythesize beta-casein mRNA (B. Binas et al., in press). Under these conditions, the proliferative rate, as indicated by the labeling index, is very low.

Table 3 summarizes effects of P108 on the differentiation of mammary epithelial cells in the presence of EGF. The continuous presence of EGF during culture caused a clear suppression of the differentiated phenotype

*Table 3.* Stimulation of functional differentiation by P108

| | PIH | EPIH | EPIH + P108 |
|---|---|---|---|
| Vacuolization index[a] | 0.69 | 0.15 | 0.60 |

Results of a typical experiment are shown.
[a] The vacuolization index is estimated as the quotient between the number of alveoli with secretory alveoli and the number of nonsecretory alveoli.

of alveolar cells. The vacuolization index was chosen as a parameter to quantitatively characterize the degree of morphological differentiation. The EGF-exerted inhibition of functional differentiation could be reversed by the additional presence of the synthetic MDGI peptide. P108 reversed the vacuolization index to the normal control level (Table 3).

EGF-dependent inhibition of ductal growth and functional differentiation have been described earlier [48]. The underlying mechanism is unclear, though most likely it is not associated with the mitogenic activity of EGF [48]. EGF probably interferes with transcriptional mechanisms involved in the maintenance of functional differentiation in the mammary gland of the pregnant mouse [49].

### *4.3. Desensitization of beta-adrenergic receptors*

The close structural relationship between MDGI and heart FABP led us to test MDGI on cardiac myocytes [40]. It turned out that in neonatal cardiac myocytes MDGI exhibited an effect that at first glance seemed unrelated to growth inhibition. These cells, when taken into primary culture, beat with a basal frequency of 140–160 beats/min. In the standard system developed by G. Wallukat et al. [50] and used for the tests, beta-adrenergic agents increase the beating rate in a characteristic dose-dependent manner, with the first response detectable at about $10^{-8}$ to $10^{-7}$ M isoprenaline. The authors found that this sensitivity can be greatly increased by pretreating the cells with L(+)-lactate [51]. In this way the cells respond to isoprenaline at $10^{-12}$ M, probably on account of an increased responsiveness or accessability of a subpopulation of $beta_2$-adrenergic receptors to the agonist. Regarding the mechanism of this sensitization, circumstantial evidence points to a metabolic pathway involving activation of phospholipase A2, the generation of arachidonic acid, and conversion of arachidonic acid to 15S-hydroxyeicosatetraenoic acid (15S-HETE) or 11S-HETE by a lipoxygenase [52,53]. This concept is also supported by findings that the supersensitivity can be induced directly with 15S-HETE or 11-HETE, but not with 15R-HETE [52,53].

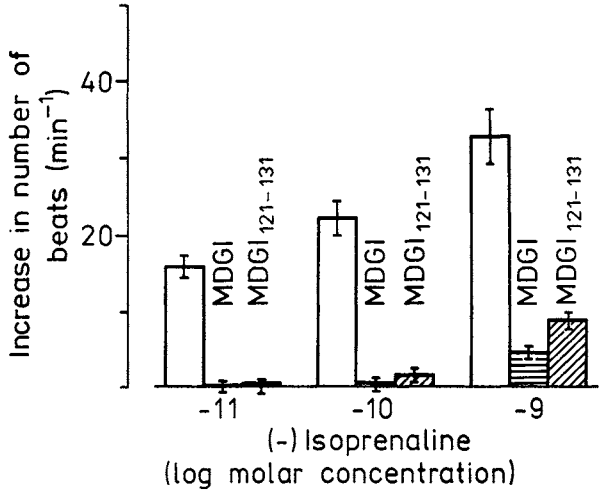


*Figure 3.* MDGI and the C-terminal peptide $MDGI_{121-131}$ inhibit beta-adrenergic supersensitivity induced by 15S-HETE. Neonatal rat cardiac myocytes were treated with 15S-HETE ($10^{-8}$M) in the absence or presence of MDGI (50 ng/ml) or the peptide $MDGI_{121-131}$ (TAVCTRVYEKQ, $10^{-7}$M) and then were stimulated with various concentrations of (−)isoprenaline. The beating frequency was recorded as described in Bullogh and Laurence [8]. The data are taken from Lettre et al. [9] or were provided by G. Wallukat (personal communication).

MDGI strikingly inhibited the induction of supersensitivity by all agents. Moreover, also the C-terminal peptide (MDGI 121-131 or the Ser-derivative P108) blocked arachidonic acid and 15S-HETE-induced supersensitivity ([40], Figure 3). This means that a mechanism of interference by MDGI subsequent to the generation of 15S-HETE is involved [40]. A summary of the effects and a schematic illustration of the current knowledge on the mechanism is depicted in Figure 4.

The data have several interesting implications. In particular, they draw the attention to the possibility that the beta-adrenergic system might be important for mammary epithelial cell growth and differentiation, and that the effects of MDGI on growth and beta-adrenergic sensitivity might have a common and physiologically important mechanism. Indeed, several reports point to an involvement of the beta-adrenergic receptors, $PGE_2$ and HETEs, for growth and developmental changes in the mammary gland [1,54,55]. The possible effects of MDGI or MDGI-derived peptides on related metabolic mechanisms may help to elucidate the mechanisms of normal proliferation and differentiation in the mammary gland. Second, one function of heart FABP could be to protect, the heart, under pathophysiological conditions, from lipoxygenase metabolites causing supersensitivity of $beta_2$-adrenergic receptors. Finally, the data indicate common targets to

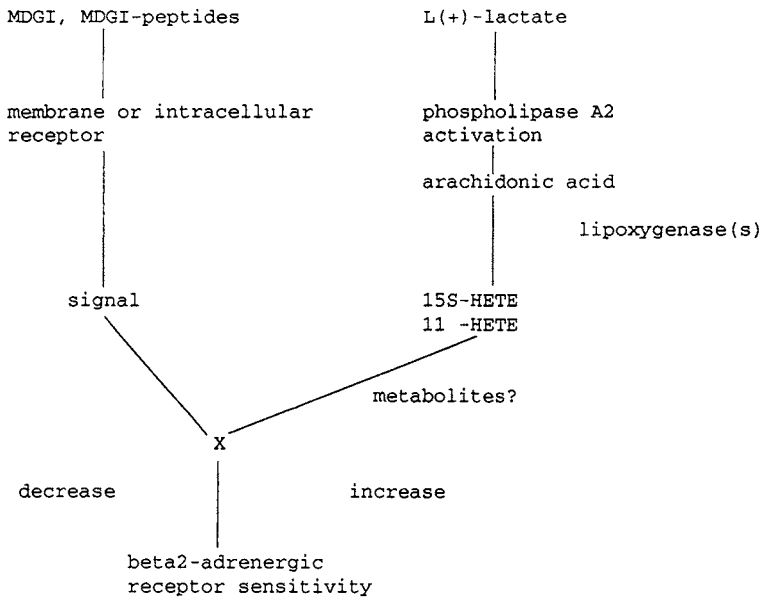


*Figure 4.* Schematic illustration of proposed steps for the interference of MDGI and MDGI-derived peptides with the induction of beta-adrenergic supersensitivity in neonatal cardiac myocytes.

MDGI and lipoxygenase products in the modulation of cellular functions. In this respect, it is interesting to note that recent data show an involvement of the lipoxygenase pathway in the signaling of growth stimulation of mammary epithelial cells [56], as well as of growth inhibition by TGF-beta [57,58], interferon [59], and TNF-alpha [60].

### *4.4. Differentiation of embryonic stem cells*

Growth factors have been suggested to play a key role in cellular proliferation, differentiation, and morphogenesis throughout the development of vertebrates. There is ample evidence that at least TGF-beta, bFGF, PDGF, and an EGF-like activity are involved in early inductive processes and in the differentiation program of the early embryo [61]. MDGI and its biologically active peptide P108 were therefore tested on embryonal stem cells (ESC) [62].

The feeder-dependent pluripotent murine ESC line BLC6 [63], when maintained under nonadhesive conditions, gives rise to the formation of so-called embryoid bodies, resembling blastocysts. After s.c. injection into syngeneic mice, the blastocysts develop into local teratocarcinomas con-

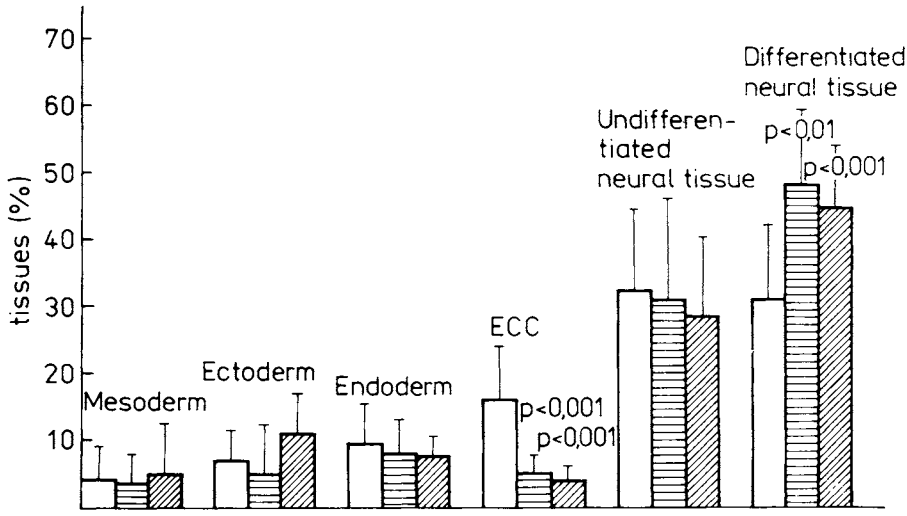


*Figure 5.* Relative volume proportions of histological structures in teratocarcinomas that arose from s.c. injection of embryoid bodies treated with MDGI. The point sampling method was used for evaluation of the histology. Empty columns = controls; horizontally hatched columns = single treatment with MDGI (50 ng/ml); cross-hatched columns = treatment with MDGI (50 ng/ml) at days 1, 3, and 4 of culture. Bars = standard errors.

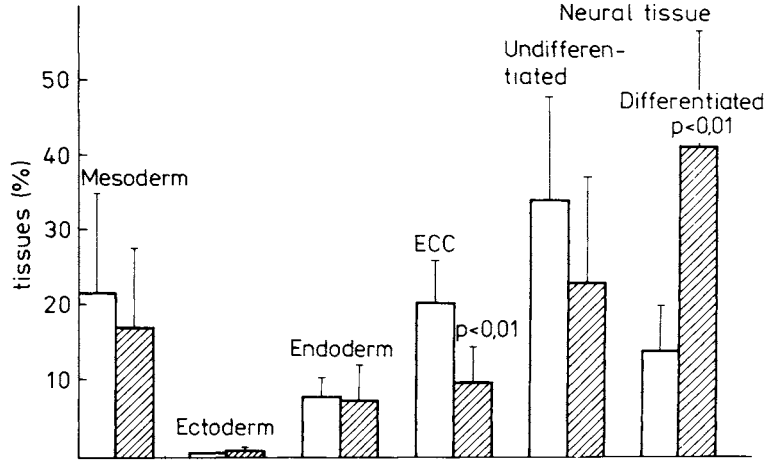


*Figure 6.* Relative volume proportions of histological structures in teratocarcinomas that arose from s.c. injection of embryoid bodies treated with the synthetic MDGI-peptide P108. Cross-hatched columns = treatment with P108 ($5 \times 10^{-6}$ M). Bars = standard errors.

sisting of undifferentiated ECC, mesodermal, endodermal, ectodermal, neuroectodermal, and differentiated neural structures. Their relative volume proportions can be estimated in histological sections by means of the point sampling method. The embryoid bodies were treated with MDGI or P108, injected into mice, and the resulting teratocarcinomas were histologically classified (Figures 5 and 6). The following results were obtained:

1. In all control and experimental groups, local tumors occurred after about 30 days with a similar efficiency rate of about 60%.
2. MDGI (Figure 5) and P108 (Figure 6) significantly reduced the relative volume proportion of undifferentiated ECC.
3. MDGI (Figure 5) and P108 (Figure 6) significantly increased the relative volume proportion of differentiated neural tissue.

The results indicate that in the C-terminus of MDGI information is buried that supports the commitment of undifferentiated ESC for neural differentiation. MDGI is structurally very close to a brain-derived FABP [64,65]. It is tempting to speculate that our findings are related to a yet unknown biological function of brain FABP.

## 5. Expression of MDGI in the mammary gland

### *5.1. Developmental regulation in the bovine mammary gland*

The MDGI expression followed on the protein and mRNA levels is dependent on the developmental stage of bovine mammary tissue. This was shown by immunochemical methods [66], by cell-free, in-vitro translation experiments [66], and by a Northern blot analysis [18]. MDGI is not detectable in virgin mammary tissue. The onset of MDGI expression was detected during pregnancy with increasing amounts towards terminal differentiation of the gland [12,66]. In the midpregnant mammary tissue, which is characterized by a high proliferation rate and ongoing differentiation, the amount of MDGI reaches 0.1% of the total protein and mRNA. Maximal MDGI expression was obtained in the lactating, terminally differentiated mammary gland with 0.5–1% of total protein and RNS [66]. MDGI is also secreted into bovine and human milk [44]. Its level increases with lactation [44].

MDGI transcripts and protein were analyzed in tissue sections from embryonic, virgin, pregnant, and lactating mammary gland [8]. In the mammary anlagen of a 5-month-old female embryo, MDGI transcripts and protein are clearly detectable. MDGI is evenly distributed over the proximal and distal epithelial cells of the mammary rudiment and the epidermis. Mesenchymal tissue was not labeled. Beta-casein expression was also found but was restricted to the mammary epithelial rudiment. The expression of beta-casein and MDGI is probably due to the presence of maternal lactogenic hormones in the embryo.

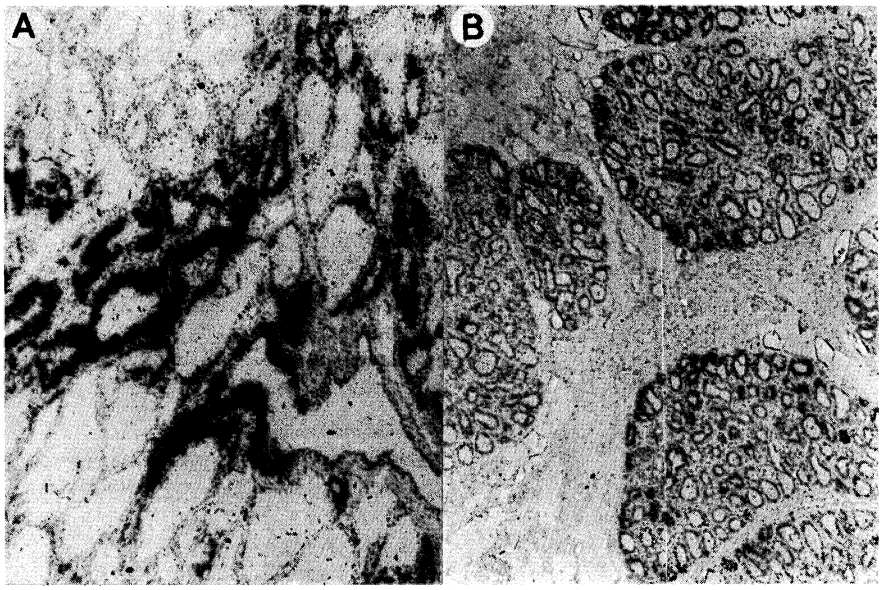


*Figure 7.* Distribution of MDGI-mRNA expression in pregnant (A) and lactating (B) bovine mammary tissue. MDGI transcripts were detected by in situ hybridization using cryostate sections hybridized with a 35S-labeled antisense RNA probe. Bright field pictures counterstained with hematoxyline are shown A: Cross section of midpregnancy mammary gland showing a lobuloalveolus embedded in connective tissue. Only alveolar epithelial cells express MDGI at increasing levels on cells bordering the connective tissue. B: Longitudinal section of terminal differentiated mammary tissue. Note the increased MDGI mRNA levels in the terminal part of a duct. (Magnification ×40.)

In the immature, resting mammary gland of the virgin animal, MDGI is not expressed. During midpregnancy, ductal and alveolar epithelial cells, in combination with myoepithelial cells, form the lobuloalveolar gland. During that time of development ductal cells express MDGI at a rather low level, whereas MDGI is clearly detectable over the alveoli.

Other cell types, such as myoepithelial cells and connective tissue, do not transcribe the MDGI gene (Figure 7). We found that early alveolar differentiation is coupled with an increase in MDGI transcription. Interestingly, in those alveolar epithelial cells bordering the connective tissue MDGI transcripts are augmented [18]. These are the epithelial cells with an increased proliferation rate that first start to transit into functional differentiation [67]. The presence of mesenchymal components has been shown to be important for the induction of limited functional differentiation [68].

The induction of MDGI expression by paracrine-acting connective tissue signals could be accompanied by a feedback reaction causing inhibition of growth and the onset of functional differentiation of the mammary epithelium. On the other hand, it might also be possible that MDGI expression

is a necessary prerequisite for the alveolar outgrowth into the regressing stroma. The terminally differentiated, lactating mammary gland is characterized by ducts branching into large secretory active lobuloalveolar structures (Figure 7). In contrast to the pregnant stage, at that time both the alveolar and the ductal epithelial cells express MDGI. Along the duct a striking increase in the level of MDGI expression was obtained with the highest levels in the terminal part. In lactation MDGI is expressed at higher levels in the ductal epithelial cells than in the alveolar epithelial cells. Myoepithelial cells and fibroblasts again do not express MDGI.

In the lactating mammary gland, MDGI expression follows an anatomical-histological gradient. In the less differentiated distal parts of the gland, the MDGI level is clearly reduced compared to the proximal regions.

In all stages of mammary-gland development, the MDGI-mRNA level parallels the protein level. This was also confirmed by an electron microscopical analysis of the intracellular distribution of MDGI in the mammary gland of different functional state [66]. Immunolabeling was associated with basal invaginations, the cytosol, and the transcriptionally active euchromatic regions of nuclei.

The apparent close parallel between the expression of MDGI and its mRNA during normal mammary gland development suggest that transcriptional control mechanisms are a major regulator. Those control mechanisms could be induced by lactogenic hormones, such as prolactin, leading to the enhanced MDGI expression necessary for local control of proliferation and differentiation.

### *5.2. Hormonal dependence of expression in mouse organ culture*

In the preceeding section we showed that local MDGI expression is developmentally regulated and is probably under the control of systemic hormones. Also, we mentioned that in monolayer cultures with primary or permanent growing mammary epithelial cells, MDGI expression is very low and could not be induced by lactogenic hormones. For example, at conditions under which HC11 cells responded to dexamethasone, insulin, and prolactin by beta-casein expression, which could be completely prevented by TGF-beta [69], the MDGI level stayed either low or unchanged at all treatments. In order to get access to the more complex mechanisms of MDGI expression, we took advantage of the organ-culture system employing explants from abdominal mammary glands of primed virgin and pregnant mice.

The mammary gland of 4-week-old virgin Balb/c-mice primed with estradiol and progesterone consists of a system of sparsely branching ducts embedded in adipose tissue. Upon cultivation with aldosterone, prolactin, insulin, and hydrocortisone (APIH medium), lobuloalveolar structures develop that exhibit cubic epithelial cells with fat vacuoles and eosinophilic secretory material in their lumina. We applied mRNA hybridization and

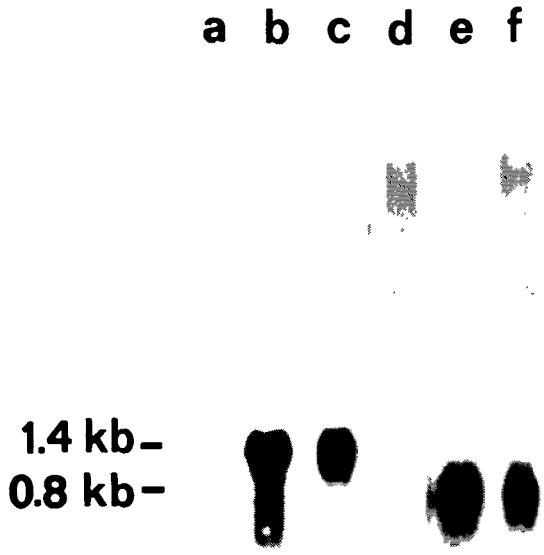


*Figure 8.* Hormone-dependent induction of MDGI expression. Northern blots probed for beta-casein (a–c) or MDGI (d–f) transcripts are shown: a,d: virgin mammary gland; b,e: lactating mammary gland; c,f: upon organ explant culture with aldosterone, prolactin, insulin, and cortisol for 9 days.

immunohistochemical techniques to follow beta-casein and MDGI expression in explants cultured with the APIH medium [Binas et al., in press]. Figure 8 shows that both beta-casein (lane a) and MDGI (lane d) transcripts, which are absent in the mammary tissue of primed virgin mice, become expressed in the terminally differentiated mammary tissue from lactating mice (lanes b and e for casein and MDGI, respectively). Transcripts of the same size (in lane c for beta-casein and lane f for MDGI) are clearly detectable in mammary gland explants after 9 days of culture in the APIH medium. Thus, local expression of beta-casein and MDGI mRNA can be induced by a combination of systemic hormones.

The cellular localization of MDGI and casein was analysed by immunohistochemistry (Figure 9). As depicted in Figure 9B, casein becomes clearly expressed upon culture in the APIH medium in alveoli, ductules, and the lumina if compared to the control (Figure 9A). For MDGI the pattern of immunostaining is different — only alveoli are immunostained (Figure 9C).

The use of organ explants from primed virgin mice for studying in vivo morphogenesis implies that lobuloalveolar development and functional differentiation cannot be separated from each other in culture.

In order to study a developmental stage associated with functional dif-

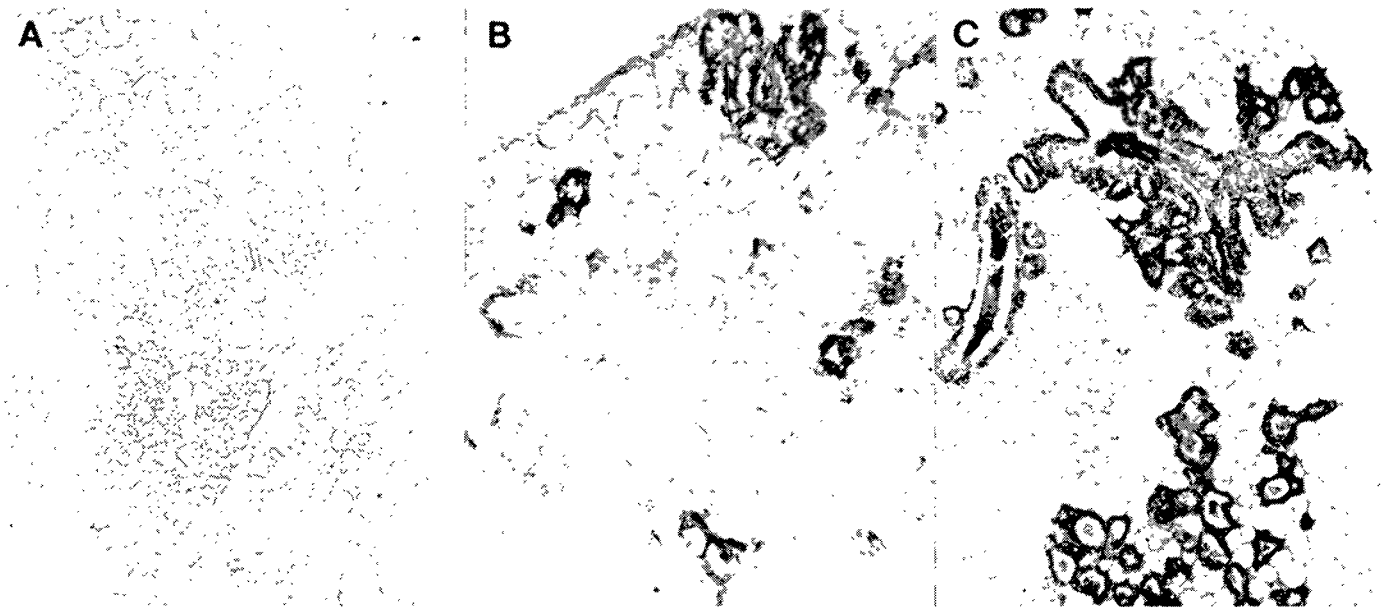


*Figure 9.* Immunohistochemical detection of hormone-dependent MDGI expression. Mammary gland explants were cultured for 9 days in the presence of aldosterone, prolactin, insulin, and cortisol. At the beginning of culture neither MDGI nor casein are expressed (A). MDGI and casein expression were visualized by the protein A gold technique in paraffin sections incubated with an anti-beta-casein antiserum (B) or with anti-MDGI antibodies (C).

ferentiation rather than with lobuloalveolar morphogenesis, we took advantage of organ explants derived from late-pregnant mice. They were cultured in a medium containing prolactin, cortisol, and insulin (PIH medium). In addition, to monitor the influence of prolactin on MDGI expression, either the hormone concentration was reduced or prolactin and cortisol were replaced by EGF (Table 4). At optimal prolactin concentrations, the beta-casein mRNA level could be even slightly enhanced, while at the same time the MDGI mRNA level clearly declined. At a reduced prolactin concentration, transcription of the MDGI gene ceased completely, while some casein expression still took place. It should be emphasized that under these conditions the typical phenotype of differentiated alveoli was maintained, although lowering the prolactin concentrations led to suppression of secretory alveolar activity (not shown). In analogous experiments, directed at maintaining the differentiated morphology of lobuloalveoli, prolactin and cortisol were substituted by EGF. Under these conditions MDGI expression is completely inhibited. However, by subsequently culturing the EI-preteated explants in the PIH medium, MDGI transcription could be reinduced and reached the PIH control, as measured by an increase in the mRNA level (Table 4).

Finally, MDGI behaves as a differentiation marker for the mammary gland, differing in some aspects from casein. The cellular patterns of expression are variable in their dependence on the developmental stage and seem to be regulated by different mechanisms. The data support a role for MDGI during functional differentiation in the mammary gland of pregnant mice. This role is at least dependent on prolactin. We are currently investigating this problem in more detail.

### *5.3. Nuclear localization*

Immunocytochemistry of Lowicryl K4M embedded sections, as well as of cryosections from lactating bovine mammary gland tissue, showed the euchromatic regions of nuclei from mammary epithelial cells to be densely labeled with the immuno-gold complex [66]. Since then other members of the FABP family have also been shown to be present in the nuclei of hepatocytes and myocytes [70,71].

In order to prove the molecular identity of the nuclear antigen, lactating mammary tissue was homogenized and a nuclear fraction was enriched. By Western blotting and immunostaining with affinity-purified anti-MDGI antibodies, only tiny amounts of MDGI could be detected — much less than expected from the immunocytochemistry. We assume that the main part leaked out during the tissue homogenization and centrifugation steps. However, by probing the Western blots of the nuclear fraction with anti-MDGI antibodies, another crossreactive, 70-kDa protein was detected (Figure 10). This protein was neither present in the soluble cytoplasmic fraction nor in the mitochondrial or microsomal fraction. It thus seems to be

*Table 4.* Prolactin dependence of MDGI expression

| Conditions in intact tissue | Relative mRNA levels | |
|---|---|---|
| | Beta casein 100 | MDGI 100 |
| d5, 5 µg/ml P; I,H | 143 | 27 |
| d5, 0.5 µg/ml P; I,H | 23 | 0 |
| d1, EI | n.d. | 0 |
| d1 EI + d2 5 µg/ml P; I,H | n.d. | 35 |

Values are expressed as the percent of the RNA level in the intact tissue before culture. Total RNA was extracted at the indicated number of days of in vitro culture and was hybridized with the $^{32}$P-labeled mouse MDGI cDNA and beta-casein probes. Northern blots were densitometrically scanned and normalized to ribosomal RNA. P = prolactin; E = 100 ng/ml EGF; I = 5 µg/ml insulin; H = 0.1 µg/gml cortisol.

truly nuclear. The 70-kDa antigen was also recognized by antibodies raised against synthetic peptides of the MDGI sequence (Figure 10). Comparing the nuclear fractions from several tissues with respect to the presence of p70 indicates the mammary gland specificity, in keeping with the MDGI distribution (Figure 10B). Preliminary experiments also indicate a DNA-binding capacity of p70 (T. Müller, unpublished data). The MDGI-related 70-kDa protein was purified by a combination of steps, including salt extraction of nuclei, ammonium sulfate precipitation, selective solubilization, passage through Q-Sepharose, mono S-chromatography, and RP-chromatography in formic acid. Several peptides comprising 120 amino acids could be sequences. They did not show any significant homology, neither with MDGI nor with any protein in a sequence data bank.

From the sequencing data it is apparent that p70 constitutes a protein that is different from MDGI. It still might be a complex of some unknown binding protein with MDGI. Alternatively, it could be an entirely different protein, sharing a structural and/or functional domain with MDGI, for example, a ligand-blinding domain common to MDGI. Taking into account its association with chromatin, purification behavior, yield, and DNA binding capacity, it is tempting to speculate about a role of p70 as a transcription factor. The ongoing cDNA cloning of p70 is expected to bring us closer to a function of this interesting relative of MDGI.

## 6. Conclusions

The tissue- and cell-specific, developmentally and hormone-dependent expression; the preferential inhibitory action on the in vitro proliferation of

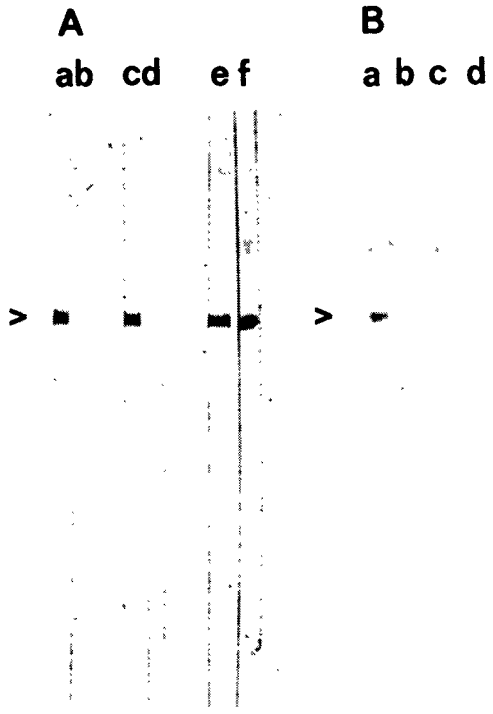


*Figure 10.* Immunological relationship between MDGI and the nuclear protein p70. Western blots are shown. A: Different anti-MDGI antibodies were tested for p70 in nuclear extracts: a, anti-MDGI antiserum; c, affinity-purified anti-MDGI-IgG; e, antibody directed against residues 69–78 of the MDGI sequence; f, antibody directed against residues 121–131 of MDGI. For control antibodies were pretreated with an excess of MDGI (lanes b and d). B: Tissue distribution of p70: Nuclear extracts from bovine lactating mammary (a), liver (b), kidney (c), and pancreas (d) were probed for p70 with anti-MDGI-IgG.

normal mammary epithelial cells; the differentiation-modulating activity in organ cultures; and the antagonistic interaction with the 'mammogenic' EGF indicate a role for MDGI in the local regulation of the growth and development of the mammary gland.

MDGI is most likely the hitherto best investigated representative of a larger family of proteins involved in the regulation of proliferation and differentiation in a wide range of tissues.

It remains to be elucidated whether different forms of MDGI, encoded by different genes, regulate different functions, such as lipid transport or growth inhibition, or the same molecule acts on different events at different stages of tissue development.

What are the functions of the most close MDGI relatives in the heart or brain? A comparison of the C-terminus of seemingly functionally unelated FABPs with MDGI reveals a strong similarity with P108 (Figure 11). It

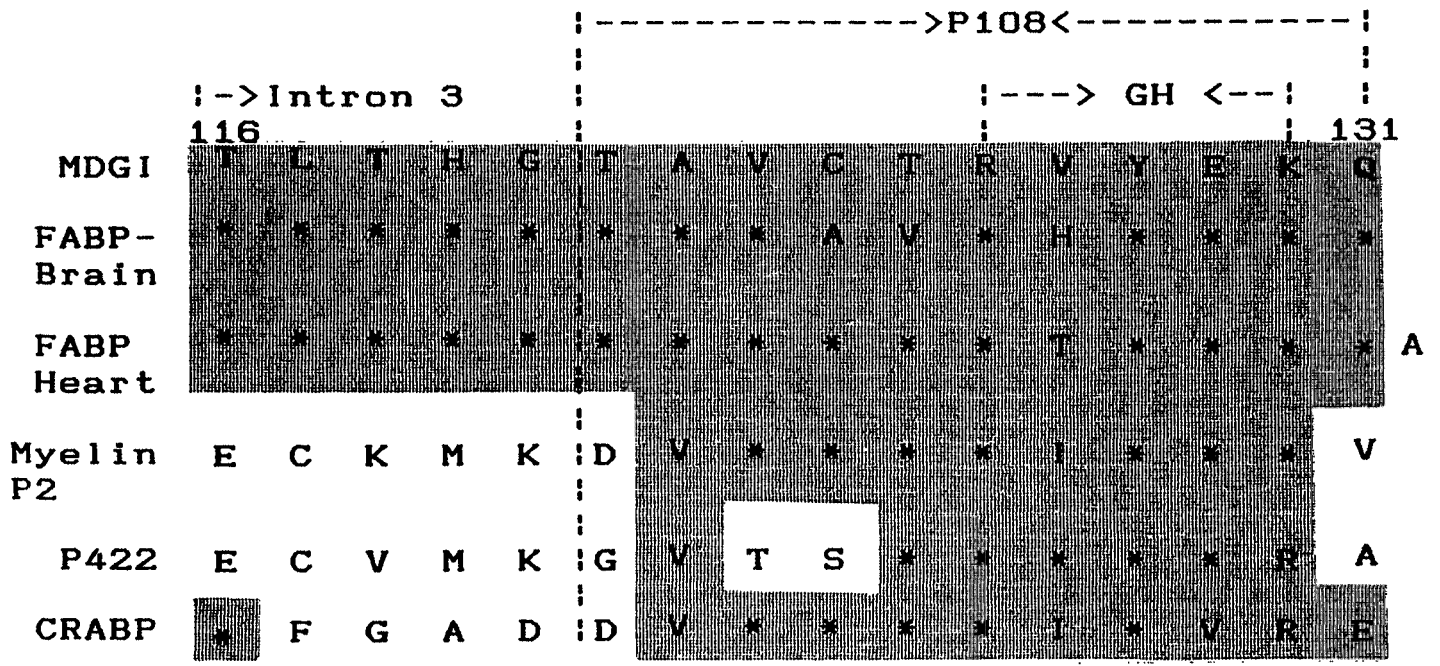


*Figure 11.* Structural comparison between C-terminal regions of MDGI-related proteins. The stretches of amino acids comprising the biologically active MDGI peptide P108 and the functional domain of bovine growth hormone are indicated. The dark field shows identical and conservatively exchanged residues.

would be interesting to test whether C-terminal peptides of the related proteins are biologically active in their respective growth assays.

In addition to the functional tests of different synthetic peptides, mutated recombinant MDGI forms deduced from MDGI and FABP-cDNA sequences can be produced in *E. coli* and tested in growth assays. In parallel, cell-transfection experiments with the different MDGI cDNA forms expressed in normal and transformed mammary epithelial cells under the control of inducible promoters should further encircle functionally important MDGI forms.

The comparison of the intron-exon structure of genes of the protein family supports a common ancestral origin [15]. Future work will show whether certain differences in the promoter regions and other regulatory elements reflect divergent evolution to accomplish specialized functions in tissue-specific growth and development. Genomic cloning of the MDGI gene will provide the prerequisite for studying transcriptional control mechanisms of MDGI expression.

Which elements direct the expression the MDGI in the mammary gland and H-FABP in cardiac tissue? What is the molecular basis for hormone-dependent MDGI expression during differentiation? What is the mechanism switching off MDGI expression in normal proliferating and mammary carcinoma cells?

We have not yet identified a pathway leading to the mechanisms underlying the growth-inhibitory or differentiation-associated activities of MDGI. It has also to be considered a primarily intracellular mechanism of action, as we have not been able to identify a membrane receptor for MDGI. What are the second messengers signaling growth inhibition and/or the induction of differentiation? In this regard, our findings about the suppression of 15 HETE-induced receptor activation by MDGI and by its C-terminal MDGI peptide provide the first intriguing idea about one possible pathway. The involvement of eicosanoids in the regulation of proliferation and differentiation has been discussed in terms of the stimulation of growth of the mammary epithelium [1].

It is exciting to presume that this pathway is affected by MDGI because it could lead us to new and physiologically important regulators of growth and differentiation.

Finally, MDGI expression is dependent on systemic hormones. Therefore, the study of its action might serve as a model indicating how the interplay between endocrine and locally acting factors controls growth and development in the normal mammary gland and to identify steps abrogated in mammary carcinoma cells.

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# 6. Roles for transforming growth factors-β in the genesis, prevention, and treatment of breast cancer

Lalage M. Wakefield, Anthony A. Colletta, Bryan K. McCune, and Michael B. Sporn

## 1. Introduction

### *1.1. The negative autocrine hypothesis revisited*

Cell proliferation is normally controlled by the opposing actions of growth stimulators and growth inhibitors. Regulated changes in this balanced system determine the rate of tissue growth during embryogenesis, and later in response to damage or altered functional demand. The negative autocrine hypothesis proposed that any defect in a normal autocrine growth-inhibitory loop could lead to tumor formation in the affected cell type by upsetting this balance [1]. The mechanistic underpinning of this hypothesis lies in the fact that cellular proliferation is a prerequisite for the fixation of deleterious mutations, and thus for tumor promotion and malignant progression [2]. Evidence for the importance of loss of negative regulation in tumorigenesis has been provided by the identification and characterization of 'tumor suppressor' genes, exemplified by the retinoblastoma gene, where loss of both alleles leads with high frequency to the development of a particular malignancy [3].

The negative autocrine hypothesis can be extended to include lesions in paracrine inhibitory interactions between two cell types. In this case, a mutation might arise in one cell type such that it fails to elaborate a regulatory protein that would normally inhibit the growth of a neighboring cell. The resulting uncontrolled proliferation of the neighboring cell would increase the likelihood of its accumulating undesirable mutations and progressing to malignancy. Furthermore, since differentiation and proliferation are mutually exclusive in many cell systems, lesions in autocrine or paracrine pathways delivering signals to differentiate could have a similar effect. This is summarized in the scheme in Figure 1. The extent to which lesions in a particular growth-inhibitory or differentiation control loop could drive tumorigenesis would depend on the level of redundancy of control loops and the dominance of the regulatory circuit considered.

Corollaries of this extended hypothesis include (1) restoration of a defective negative control loop should restore normal growth control, (2) substitution of a defective negative control circuit with an alternative one

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

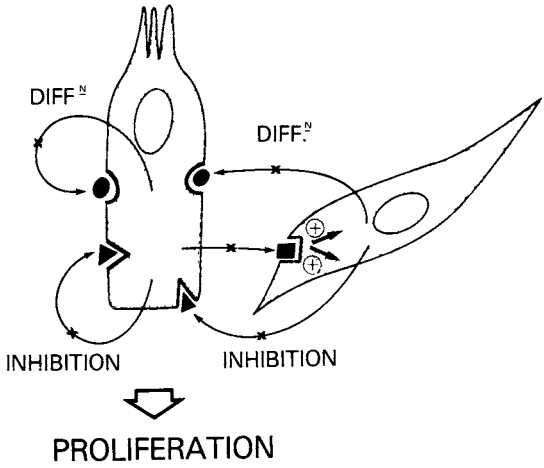


*Figure 1.* The negative autocrine hypothesis extended. In addition to the lesions in negative autocrine signaling paths proposed in the original autocrine hypothesis, lesions in paracrine signaling paths for growth inhibition or for differentiation could also lead to increased proliferation of the target epithelial cell. Furthermore, the breakdown in paracrine communication could be due to defects either in the epithelial cell itself, if this normally acted on the neighboring stromal cell to induce or enhance such communication, or it could be due to defects in the stromal cell.

should also restore normal growth, and (3) augmentation of a normal negative control mechanism may serve to counterbalance the tumorigenic effect of a positive autocrine lesion, such as overexpression of a growth factor or activation of an oncogene. In every case, the negative signal could be delivered in either an autocrine or a paracrine manner.

Members of the transforming growth factor-β superfamily have emerged as key regulators of many aspects of cellular physiology, with potent effects on cell growth and differentiation. In particular, TGF-βs are strongly growth inhibitory for many epithelial cell types. In this review we shall consider potential roles for members of the TGF-β family in regulatory interactions in the breast, using the conceptual framework outlined above. The evidence for TGF-β family members as endogenous negative regulators of mammary gland development will be presented, and the question of whether defects in the TGF-β control loops contribute to mammary carcinogenesis will be examined. Finally, the potential for pharmacological manipulation of endogenous TGF-β levels for the chemoprevention or treatment of breast cancer will be discussed.

## 2. The biology of transforming growth factors-β

For comprehensive reviews of TGF-β biology, the reader is referred to Roberts and Sporn [4], Massagué [5], and Moses et al. [6]. Here we will

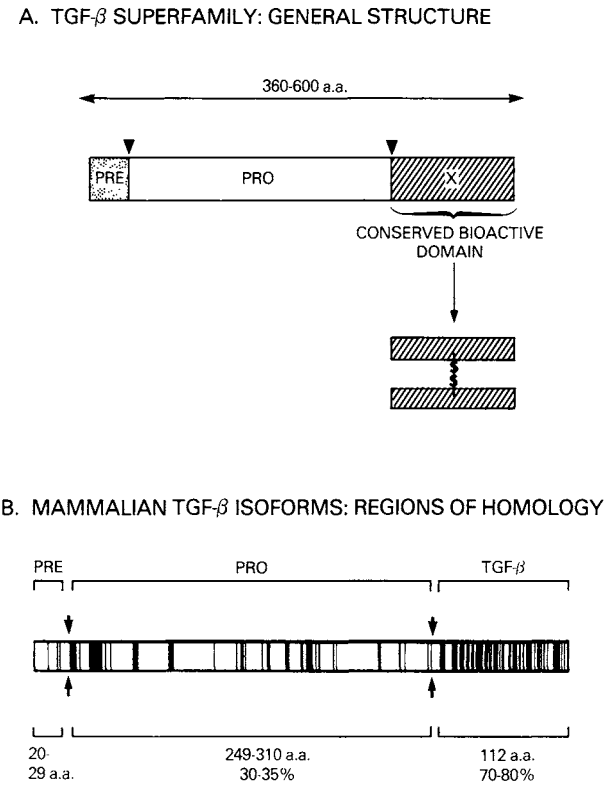


*Figure 2.* The structure of TGF-β superfamily proteins. The bioactive domain of the TGF-β superfamily members is a conserved region at the C-terminus of a longer precursor protein, containing a motif of seven cysteines that is absolutely conserved between all family members. In most cases, the active peptide is a disulfide-linked dimer of this conserved region. The three mammalian TGF-βs are highly homologous to each other in this C-terminal domain but show more sequence divergence in the precursor pro region. The black bars indicate regions of sequence identity between TGF-β1, -β2, and -β3, and the percent identity in each domain is given below. More distant family members, such as the inhibins and the bone morphogenetic proteins, show no homology to the TGF-βs or each other in the pro region.

only give an overview, concentrating on aspects that are most relevant to understanding potential roles for TGF-β in the breast.

## *2.1. The TGF-β family of proteins*

The TGF-βs are a subgroup within a superfamily of genetically related proteins involved in the regulation of growth, differentiation, and function in diverse organisms. The general structure of the protein precursor for TGF-β superfamily members is shown in Figure 2A, together with the regions of sequence identity between the three mammalian TGF-β isoforms,

TGF-βs 1, 2, and 3 (Figure 2B). The biologically active TGF-β species is a 25-kDa disulfide-linked dimer of the highly conserved C-terminal region, while most of the sequence diversity lies in the precursor 'pro' region.

The three mammalian TGF-βs that have been identified to data appear to act through an overlapping set of cell surface receptors [5]. In most in vitro assays they have essentially identical biological activities, although a few situations exist where this is not the case [7]. Thus, the significance of the multiple subtypes may lie less in intrinsically different biological activities and more in the potential for differential regulation. The promoter regions of TGF-βs 1–3 are very different in structure [8], and there is evidence for differential regulation of the subtypes at the post-transcriptional level too [9–11]. In particular, the considerable sequence divergence in the precursor pro region may affect the tissue targets and bioavailability of the particular TGF-β subtype (see Section 2.3).

Immunohistochemistry and in situ hybridization studies indicate distinct but overlapping patterns of expression of the individual TGF-β subtypes in different tissues, but virtually all tissues in the developing embryo and the adult animal express detectable levels of at least one of the isoforms [12–14]. Similarly, cell-surface TGF-β binding proteins/receptors are expressed on nearly all cell types, at least in vitro [5]. Thus the ligand–receptor system is essentially ubiquitous.

### 2.2. *Biological activities of TGF-βs*

Experiments in vitro suggest that TGF-βs are highly multifunctional and are probably key components in cellular signaling paths regulating and integrating cell growth and differentiation in many tissues and organ systems. As is emerging for other growth factors, the effects of TGF-βs depend critically on the nature of the cell, on its current state of differentiation, and on environmental influences, such as the composition of the extracellular matrix and the spectrum of other growth factors acting on the cell at the same time [15]. The change of state induced in the cell by TGF-β is an integrated function of all these parameters. Thus results obtained in vitro should be extrapolated with extreme caution to the complex in vivo situation, and preferably should be confirmed by suitable in vivo experiments.

Some important general features of TGF-β activities are given below.

1. *Proliferation.* The TGF-βs are strongly growth inhibitory for most epithelial cells and cells of the hematopoietic system, while being growth stimulatory for many cells of mesenchymal origin [reviewed in 4 and 6]. For some cells, TGF-β may either stimulate or inhibit cell growth, depending on the other growth factors present at the same time or the growth substratum [16]. Growth inhibition by TGF-β is generally reversible, unless coupled to terminal differentiation [17]. However, TGF-β may be associated with or may drive apoptotic cell death in some systems [18,19].

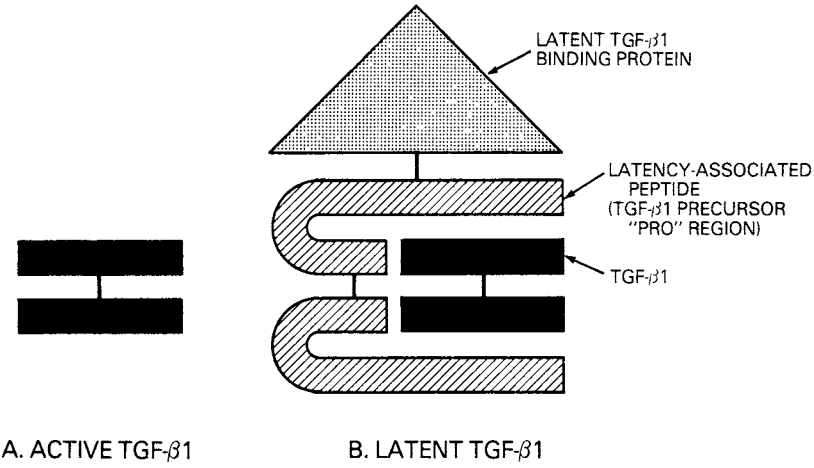


*Figure 3.* The structure of the latent TGF-β1 complex. Latent TGF-β1 (235 kDa) is composed of active TGF-β (25 kDa), noncovalently associated with a dimer of the precursor pro region (75 kDa). The pro region dimer confers latency on the TGF-β. In most cases, the pro region is disulfide bonded to a genetically unrelated glycoprotein (~135 kDa) of unknown function.

2. *Differentiation.* TGF-βs induce the expression of the differentiated phenotype in some cells and block it in others [4]. In some instances, they may act as a switch, turning on a differentiation program in progenitor cells but turning off the same functions in the mature differentiated cell [20,21].

3. *Function.* TGF-βs can regulate various aspects of the differentiated function of many cell types [4]. As with its effects on differentiation, the effects on function can be totally independent of any changes in cell proliferation.

### *2.3. Bioavailability of TGF-βs*

As noted in Section 2.1, the biologically active form of the TGF-βs is a disulfide-linked 25-kDa dimer of the C-terminal region of the precursor. However, most cells in culture secrete TGF-βs in latent forms that cannot bind to the cell-surface receptors without prior activation in vitro [22–26]. The latent form of TGF-β1 is a high molecular weight complex with three components (Figure 3). The dimeric TGF-β remains noncovalently associated with its precursor pro region after biosynthetic cleavage, and the folding of the pro region around the active TGF-β renders the TGF-β latent. A genetically unrelated 'latent TGF-β binding protein' is disulfide-bonded to one subunit of the pro region dimer [27,28]. The function of this binding protein has not yet been determined, but it may regulate the biosynthesis, targetting, or activation of the latent complex. Some cells make a smaller latent complex that lacks the binding protein [29]. The latent complexes of

TGF-β2 and TGF-β3 have not been fully characterized but are probably structurally similar to those of TGF-β1. However, the divergent sequences of the pro regions may cause differential targetting of the isoforms to different tissues or may affect the ability to be activated.

In vitro, latent TGF-βs may be activated by any perturbation that significantly disrupts the tertiary structure of the complex, such as exposure to chaotropic agents or extremes of pH, or the action of certain proteases or glycosidases [25,30,31]. The mechanism of activation in vivo is uncertain but appears to be highly complex. It may require the cooperation of two distinct cell types [32], and roles for plasmin [33] and the IGF-II/mannose-6-P receptor are indicated in some systems [34]. The nature of the activation mechanism in vivo and the circumstances under which it operates represent major gaps in our understanding of TGF-β physiology.

A number of proteins that can bind active TGF-βs have been identified in the extracellular matrix or extracellular fluid. Matrix binding proteins, such as decorin, biglycan, heparin, fibronectin, laminin, and type IV collagen, may sequester the active growth factor and modulate its availability to the cell, possibly acting as extracellular storage depots for active TGF-β [35–37]. Other binding proteins in the extracellular fluid, such as soluble beta-glycan, alpha-2-macroglobulin, and alpha-fetoprotein in the fetus, may transport the active TGF-β to distant tissues for delivery or degradation [38–40]. Indeed, active TGF-β1 is rapidly cleared from the circulation [41], whereas latent TGF-β1 has a much longer plasma half-life [42]. Thus active TGF-β probably acts locally in an autocrine or paracrine fashion, whereas in its latent form TGF-β may be able to mediate longer range interactions between distant target organs.

The possible interrelationship between the various forms of TGF-β is shown in Figure 4. Regulation of TGF-β bioavailability through binding to these various binding proteins is extremely complex, and some of the binding proteins (e.g., decorin) inhibit binding of TGF-β to its receptor while others (e.g., alpha-fetoprotein) do not. Furthermore, alpha-2-macroglobulin has a tenfold higher affinity for TGF-β2 than for TGF-β1, so there is likely to be differential binding of the TGF-β subtypes to various extracellular binding proteins, and this may contribute to the different biological activities of the subtypes in vivo [43]. A very important experimental consequence of these observations is that the identification of TGF-βs in a particular tissue by immunohistochemistry or biochemical extraction should not necessarily be equated with the presence of bioavailable TGF-β.

### *2.4. Mechanism of TGF-β action*

Three major cell-surface binding proteins for TGF-βs have been identified, two of which are thought to be bona fide receptors [for a review, see 5]. Little is known about the signal transduction paths, but binding of TGF-β to cells clearly has important effects, both on gene expression and on

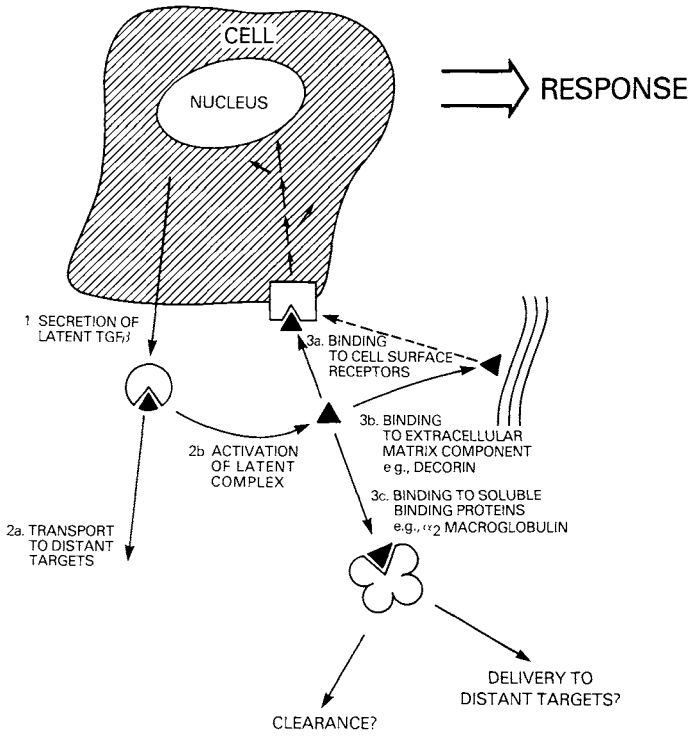


*Figure 4.* Regulation of the bioavailability of TGF-βs. Latent TGF-β secreted by a given cell may be transported to distant sites or may be activated locally, by as yet unknown mechanisms. The active TGF-β may either bind to the cellular receptors and elicit a biological response, or it may be bound and sequestered by extracellular matrix components or soluble binding proteins. Its fate after binding to these components is not clear. TGF-β bound to extracellular matrix components may be released slowly for later action, and TGF-β bound to soluble binding proteins, such as alpha-2-macroglobulin, may be cleared and degraded.

cytoplasmic events, such as message stabilization, translation, and post-translational modification of proteins. TGF-βs appear to have particularly profound effects on the expression of extracellular matrix proteins and the enzymes involved in their degradation [5,44]. Indeed, many of the biological effects of TGF-βs are thought to be mediated in part by alterations in the composition or extent of extracellular matrix. Effects on the expression and activities of protooncogenes and transcriptional regulators are also likely to be important [44,45], and recently provocative links have been established between the actions of TGF-β and the tumor suppressor gene, Rb [46].

## 3. Roles for TGF-βs in normal breast physiology

It has been observed in the mouse that, even during periods of peak growth and development of the mammary gland, >95% of the glandular epithelium

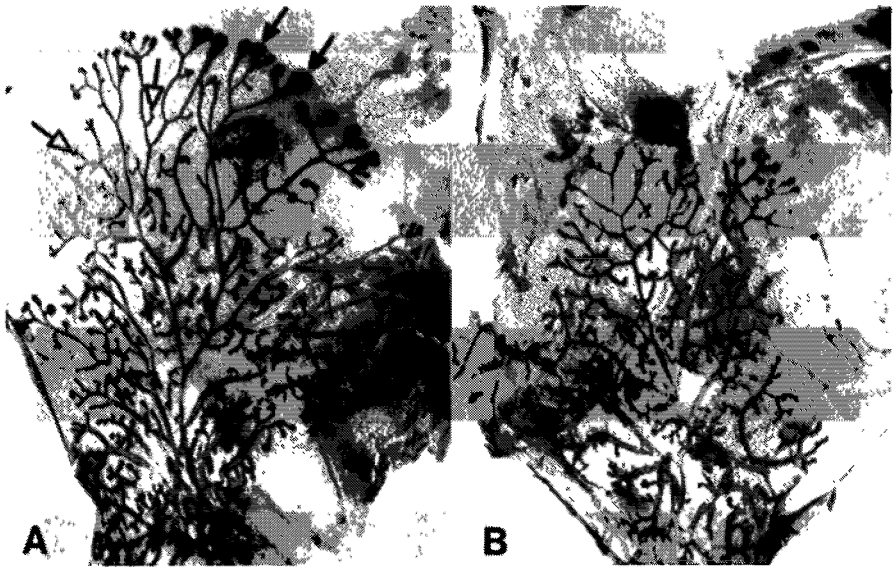


*Figure 5.* Photomicrograph showing the effect of TGF-β on ductal growth in the mouse mammary gland. Five-week-old C57 female mice were treated for 4 days with an EVAc implant containing 543 ng of TGF-β. A: Control. Closed arrows point to endbuds at the ductal growth front that have grown around and past a BSA-containing implant (*). Blunt-tipped growth-quiescent, terminal ducts (open arrows) can be seen on either side of the endbud array and are also prominent in the middle of the gland, where endbud growth is normally restricted (×5). B: Treatment (gland is contralateral to the pictured control). EVAc implant containing TGF-β (*) caused regression of endbuds and inhibition of ductal growth (×5). (Reprinted with permission from G.B. Silberstein and C.W. Daniel (1987) Science 237:291–293. Copyright 1987 by the American Association for the Advancement of Science.)

is quiescent, despite the presence of high levels of mitogens, and the fact that the quiescent epithelium retains the capacity to proliferate and form new ducts when transplanted onto cleared mammary fat pads [47]. These observations implicate an endogenous growth inhibitor, which must normally prevent epithelial proliferation, except in the region of the developing endbuds [47]. In this way, ductal hypertrophy is prevented and clear spaces are maintained between mature ducts for the subsequent development of secretory alveoli. The potent inhibitory effects of TGF-βs on many normal epithelial cells made this protein family a plausible candidate for the endogenous inhibitor in the mammary gland.

### *3.1. Effects of exogenous TGF-β on mammary development in vivo*

TGF-β1 implanted in the developing mouse mammary gland in slow-release plastic pellets had a potent inhibitory effect on the growth and morphogenesis of the mammary ductal tree (Figure 5) [48]. Endbuds regressed, and

epithelial DNA synthesis and ductal development were inhibited. The effect of TGF-β1 was reversible and no cytotoxicity or dysplasia were observed over extended periods of treatment. The morphology of TGF-β1-inhibited ducts was similar to normal quiescent ducts in untreated glands, indicating that TGF-β1 had many of the properties predicted for the endogenous natural growth regulator.

Similar experiments in pregnant or hormone-treated mice showed that the growth-inhibitory effect was highly cell specific [49]. There was no inhibition of development of the lobular-alveolar secretory structures in pregnant or hormone-primed animals. Inhibition was only observed in the rapidly proliferating epithelium of the mammary endbuds, and not in the surrounding stromal tissues, nor was there inhibition of the maintenance DNA synthesis in the lumenal epithelium of the ducts. Further analysis indicated that exogenous TGF-β stimulated the stromal cells surrounding affected endbuds to deposit glycosaminoglycans and collagen, suggesting an important role for endogenous TGF-β in the organization of periductal stroma [50]. Matrix deposition by the stromal cells was dependent on the presence of endbud epithelium, since no stimulation of periductal matrix synthesis was observed, nor was there increased synthesis in stromal cells lying near the TGF-β implant but not adjacent to the endbud. This indicates the highly cell-type specific nature of the effects of TGF-β in vivo and the importance of interactions between distinct cell types in determining the response. Interestingly, TGF-βs are highly expressed during embryogenesis in tissues where branching morphogenesis is occurring, such as the lung and salivary gland [51]. Localization and dynamics of extracellular matrix deposition in the postpartum development of the mammary ductal system and in the development of these embryonic structures are similar [50]. Thus TGF-βs may play critical regulatory roles in organizing this type of development in a number of organ systems.

### *3.2. Evidence for endogenous TGF-β in the mammary gland*

The most comprehensive analyses of TGF-β expression in the mammary gland have been performed in the mouse [52]. Northern blots indicated expression of TGF-β1 mRNA at high levels during all developmental stages except lactation. In situ studies in 5-week-old animals localized this mRNA to the epithelium of ducts and endbuds, and to adjacent stromal cells. Complementary immunohistochemical studies showed intense staining for TGF-β1 in the extracellular matrix surrounding growth-inhibited ducts, but not around ductal endbuds or small lateral branches. This staining pattern was consistent with the local loss of extracellular TGF-β being important in allowing endbud development and lateral branching. The in situ studies suggested that the extracellular TGF-β protein could be either stromal or epithelial in origin. Interestingly, in midpregnancy animals, the fibrous matrix around the mature ducts stained strongly for TGF-β1, whereas the

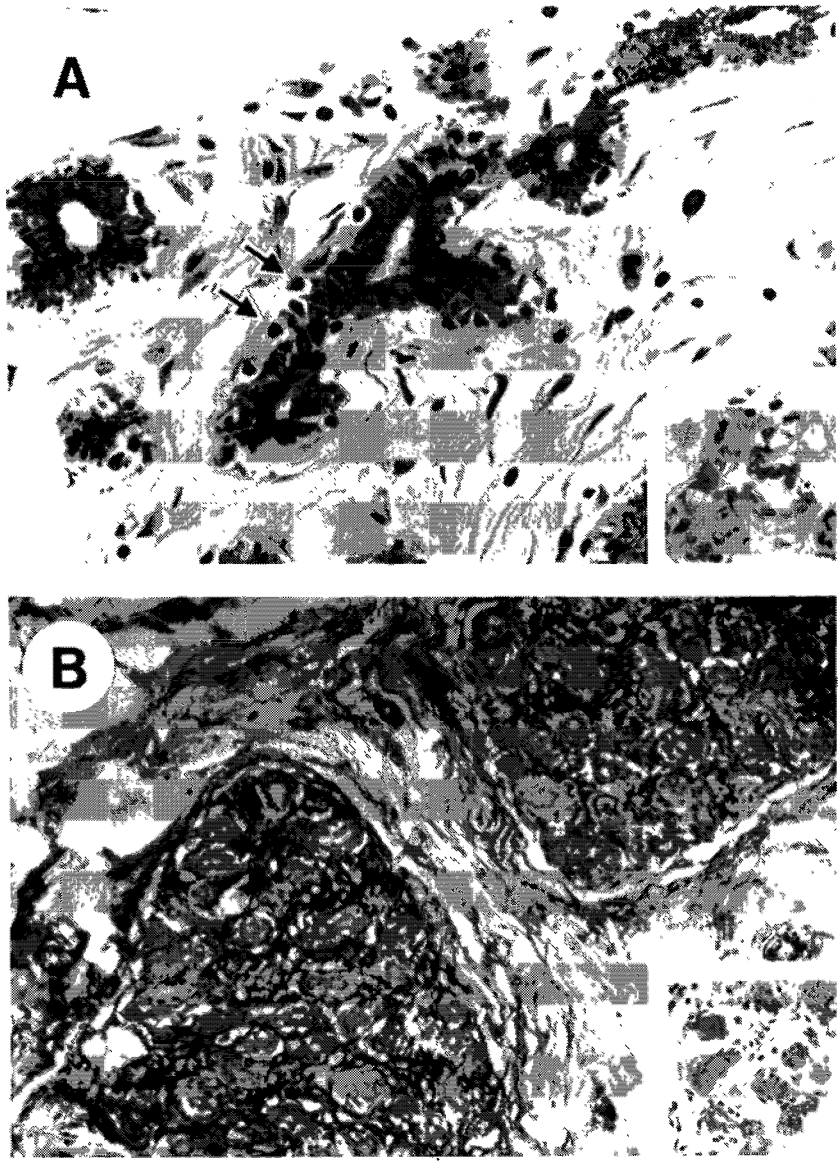


*Figure 6.* Immunolocalization of TGF-βs in the normal human mammary gland. A: Section stained with antibodies to TGF-β1. Strong cytoplasmic reactivity is seen in the epithelium of this lobule. Myoepithelial cells (arrows) and stromal fibroblasts show minimal or no reactivity. Antibodies to TGF-β2 and -β3 react with a similar distribution (not shown). Inset: Control showing no staining with TGF-β1 antibodies preincubated with the immunizing peptide. B: Section stained with TGF-β1 antibodies that recognize an extracellular conformation of TGF-β1 and -β3. Positive staining is associated with the intralobular extracellular matrix. No staining is present in the interlobular stroma. Inset: Non-immune rabbit serum control. (Immunoperoxidase with hematoxylin counterstain. A: 500×; B: 160× magnification)

matrix surrounding the secretory epithelium of alveoli stained lightly or not at all, suggesting that the loss of extracellular TGF-β may also be necessary to allow secretory differentiation to occur. By contrast, strong intracellular staining for TGF-β1 was observed in the cytoplasm of rapidly dividing epithelial cells in the endbuds in 5-week-old animals, and there was scattered staining in nondividing ductal cells. This intracellular staining appeared to be associated with putative stem cells throughout the gland. The authors proposed that, in this case, TGF-β1 might be important in maintaining the stem cells in a pluripotent, undifferentiated state.

Human studies have been limited to the nonpregnant, nonlactating adult breast, but broadly similar expression patterns for TGF-β are seen. Immunohistochemical studies have demonstrated the presence of all three mammalian TGF-β subtypes in the normal breast [53]. TGF-β1-3 were detected intracellularly in the epithelium of lobules and ducts (Figure 6A). Antibodies to the TGF-β1 and β3 precursor pro region showed similar staining, suggesting that the TGF-β is present either as the unprocessed precursor or in the biologically latent form. Little or no cytoplasmic staining for TGF-βs was observed in the stromal fibroblasts, myoepithelial cells, fat, or interlobular connective tissue. Extracellular staining for TGF-β1 was observed in the specialized intralobular stroma and the connective tissue surrounding extralobular ducts (Figure 6B).

It should be noted that the antibody that gave the extracellular staining pattern recognizes an epitope that is only exposed in extracellular TGF-β, presumably reflecting a conformational change in the molecule or altered binding to other components [54]. The functional significance of this extracellular conformation and the bioavailability of this form of the molecule are not known. As yet, no in situ data are available for the human breast, so the source of the extracellular TGF-β is not clear. By analogy with the mouse mammary gland, it could come from either epithelial or stromal cells. However, the staining patterns, with TGF-β staining rimming the epithelial component in a narrow band, suggest either epithelial synthesis or epithelium-dependent stromal synthesis. A diagram summarizing the immunohistochemical distribution of TGF-βs in the human mammary gland is given in Figure 7. This type of staining pattern is not seen in the human prostate, so it is not a universal feature of glandular epithelia and may indicate roles of TGF-βs that are specific to the breast (our unpublished observations). The simultaneous presence of TGF-βs 1–3 in the epithelium either indicates as yet unknown differences in their biological activities on mammary tissue or reflects the need for a high level of redundancy in this particular control system in the mammary gland. In the mouse, the different subtypes appear to be differentially expressed during the various stages of mammary gland development, which could be indicative of different biological activities (G. Silberstein and C. Daniel, personal communication). It will be interesting to determine how expression in the human mammary gland changes with hormonal, developmental, and functional status.

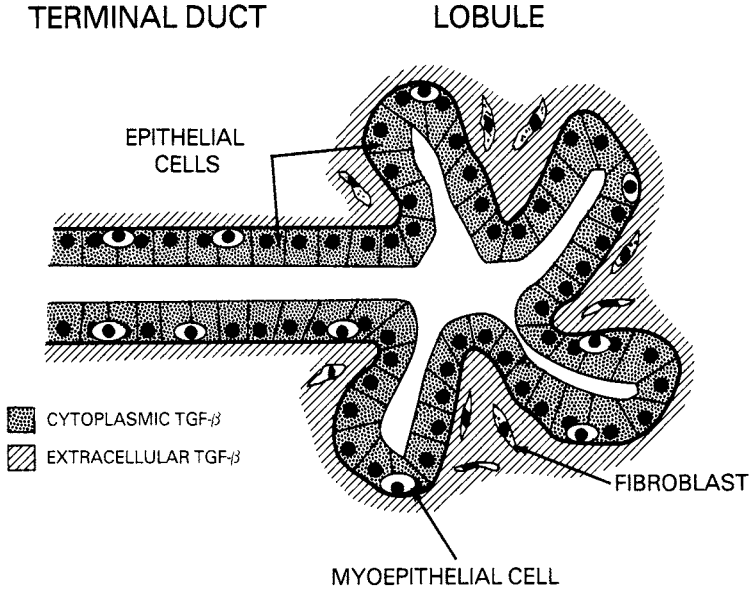


*Figure 7.* Diagrammatic representation of TGF-β distribution in normal human breast (from immunohistochemical studies).

### *3.3. TGF-β effects on normal mammary cells in vitro*

Normal human mammary epithelial cells (HMEC) derived from reduction mammoplasties or from morphologically normal areas of mastectomy tissue respond to TGF-β by assuming a flattened elongated morphology and showing extensive growth inhibition when cultured on plastic surfaces under serum-free conditions [55–57]. There appears to be some individual variation in the degree and sensitivity of the response, but generally inhibition was seen at relatively low TGF-β doses ($ED_{50}$ 1–20 pM), and >80% of the cell population was inhibited at maximal doses [55,57]. Residual mitogenesis in cultures that were not completely inhibited did not appear to be due to TGF-β resistant variants in the population, since resistance was not maintained through a second passage [55]. Growth of rat mammary epithelial cells in vitro was stimulated by anti-TGF-β antibodies, suggesting that a negative autocrine loop involving endogenous TGF-β was operating in those cells [58].

The importance of the cellular environment in the response to TGF-β is shown by experiments in which human mammary epithelial cells were cultured in a type I collagen gel matrix, with the matrix either adhering to a plastic substratum or floating in the medium [56]. Intriguingly, under these conditions the growth-inhibitory response to TGF-β was lost. In the floating collagen gels, HMEC produce three-dimensional branching structures, just

as they do when cultured on fat pads. TGF-β treatment prevented the formation of radial extensions from the floating cell mass, while having no effect on overall levels of DNA synthesis. The inhibition of the formation of radial extensions in this culture system may be analogous to the inhibition of lateral branching and ductal development seen with TGF-β in the whole animal [49].

Taken together, these results in vitro suggest that mammary epithelial cells are potentially sensitive to the growth-inhibitory effects of TGF-β, but that growth inhibition is only observed in the context of a particular cellular architecture or microenvironment. This may provide a rationalization for the observation that exogenous TGF-β in vivo is only growth inhibitory for the epithelial cells of the ductal endbuds and not for epithelia in other areas of the mammary gland, since these are invested in a different extracellular matrix and in communication with different cell types.

### *3.4. Effects of TGF-β on mammary differentiation*

Messenger RNA levels for TGF-βs 1–3 were decreased in the mouse mammary gland during lactation, and no TGF-β1 protein was observed in secretory structures, suggesting that a decrease in TGF-β levels might be necessary to allow secretory differentiation to occur [52] (G. Silberstein and C. Daniel, personal communication). Indeed, direct effects of TGF-β1 on the differentiation of isolated mammary epithelial cells have been demonstrated in vitro. TGF-β treatment of HC11 mouse mammary epithelial cells antagonized the induction of β-casein synthesis by dexamethasone and prolactin [59]. The effect was selective, since there was no change in overall protein synthesis, and it was not simply a consequence of the TGF-β-induced growth inhibition. Furthermore, TGF-β decreased synthesis of β-casein in cells that had already been induced to differentiate. This is consistent with roles for TGF-β in preventing secretory differentiation or in switching off lactation. Conversely, TGF-β upregulated the expression of milk-fat globule antigen/epithelial membrane antigen in HMECs, indicating that its effects on differentiated markers are complex and may depend on the preexisting state of differentiation or the specialized nature of the target epithelium [60]. Although there is no experimental evidence to date, it is likely that TGF-β may also have indirect effects on mammary epithelial differentiation, through effects on the underlying stroma, possibly involving changes in matrix production.

In summary, all three mammalian TGF-β isoforms are present in the normal mammary gland, and experiments both in vitro and in vivo point to roles for TGF-βs in regulating growth and function. The TGF-βs probably have locally different effects depending on the target cells and the stage of development or maturation. However, the combined evidence suggests an important role for TGF-β1 in the periductal fibrous stroma in inhibiting local proliferation of epithelial cells, thereby suppressing lateral branching and

ductal hyperplasia. Other less understood roles in maintaining ductal stem cell populations and regulating secretory differentiation are also likely. Since both stromal and epithelial cells express TGF-β1 mRNA in vivo, TGF-β action could be either autocrine, paracrine, or both in this system, and there is potential for reciprocal mesenchymal-epithelial communication via TGF-βs. Definitive proof of the roles of endogenous TGF-βs in mammary physiology awaits the results of in vivo experiments with TGF-β antagonists or transgenic knockout experiments.

## 4. TGF-βs and malignancy of the breast

The foregoing evidence suggests important autocrine or paracrine roles for TGF-βs in inhibiting the proliferation of mammary epithelial cells in the normal breast. Thus it is reasonable to ask whether lesions in TGF-β inhibitory paths might contribute to mammary carcinogenesis.

### *4.1. Lessons from other organ systems*

A number of studies in vitro have suggested that neoplastic progression in other organ systems is accompanied by a loss of responsiveness to growth inhibition by TGF-β, as predicted by the negative autocrine hypothesis. Increased resistance to TGF-β accompanied the in vitro neoplastic progression of rat tracheal epithelial cells [61] and *ras* transfection of hepatocytes [62]. In some instances, the inhibitory response to TGF-β was actually transformed to a growth stimulation in the more malignant cells [63].

However, other experiments have suggested that these results may be artefacts of in vitro culture systems. Normal, preneoplastic, and neoplastic rat hepatocytes generated in vivo in a Solt–Farber model were all sensitive to growth inhibition by TGF-β in primary culture, although at later times in culture a proportion of the hepatocytes from the normal or nodular livers became resistant to TGF-β [64]. Similarly a literature survey of the responsiveness of >50 established human tumor cell lines of epithelial and hematopoietic origin indicated that over half retained sensitivity to growth inhibition by active TGF-β [65]. However, in most cases the ability of cells to respond to latent TGF-β was not assessed, so it is possible that some tumor cells have defects in the mechanism for activation of latent TGF-β. Overall the results tend to be inconclusive, but suggest that loss of response to TGF-β may not be an obligatory step in tumorigenesis in most tissues. Retinoblastomas are a possible exception to this rule, since all retinoblastoma cell lines have lost TGF-β receptors [66].

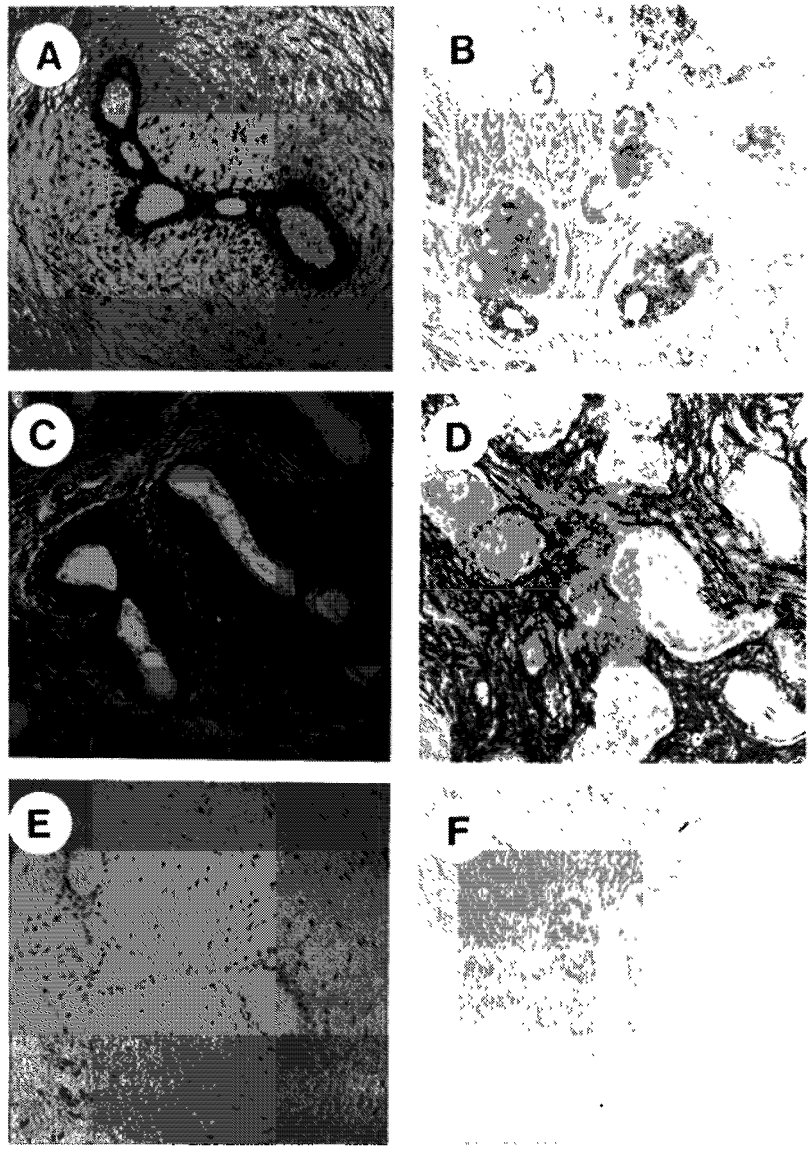


*Figure 8.* Immunolocalization of TGF-βs in human fibroadenoma (A,C,E) and infiltrating ductal carcinoma (B,D,F). A, B: Sections stained with anti-TGF-β3 antibodies show immunoreactive TGF-β3 localized predominantly to the cytoplasm of the epithelial component in both fibroadenoma (A) and carcinoma (B). Antibodies to intracellular TGF-β1 and TGF-β2 show similar staining patterns in these epithelia (data not shown). C,D: Antibodies that recognize extracellular TGF-β1 or TGF-β3 show a zone of extracellular matrix reactivity in the stroma surrounding the epithelial component of fibroadenoma (C), and widespread reactivity in the desmoplastic stroma of infiltrating ductal carcinoma (D). E,F: Controls stained with anti-TGF-β3 antibodies blocked with cognate peptide (E) or nonimmune rabbit serum (F). [Immunoperoxidase with hematoxylin (A,C,E) or methyl green (B,D,F) counterstain. A,B,D,E,F: 160×; C: 80× magnification]

*4.2. Is TGF-β implicated in mammary tumorigenesis?*

**4.2.1. Alterations in TGF-β production.** A detailed immunohistochemical study, using a large battery of anti-TGF-β antisera, indicated little difference in TGF-β expression patterns in the epithelia of normal human breast when compared with fibrocystic change, epithelial hyperplasia, sclerosing adenosis, fibroadenoma, cystosarcoma phylloides, and several breast carcinoma variants [53]. Figure 8 shows the distribution of immunoreactive TGF-βs in a fibroadenoma and an infiltrating ductal carcinoma. TGF-βs 1, 2, and 3 were present intracellularly in most mammary epithelia, including the carcinomas (Figure 8A and 8B). The only exception among the carcinomas was the epithelia within mucin pools in mucinous carcinoma, which did not stain (not shown). As with the normal human breast, little or no TGF-β was observed intracellularly in stromal cells. However, there was particularly strong staining for TGF-β1/3 in the extracellular matrix of the desmoplastic stroma (Figure 8D), suggesting that TGF-βs may play a role in the induction or maintenance of the desmoplastic response (see Section 5.1). Two other lesions characterized by abnormal proliferation of stromal cells, fibroadenoma (Figure 8C), and cystosarcoma phylloides (not shown) also showed intense extracellular TGF-β staining associated with a positive epithelial component. With the exception of this strong desmoplastic staining, the data generally indicated that altered patterns of expression of TGF-βs with respect to isoform and distribution between epithelium and stroma were not a major feature of most benign or malignant breast lesions. The data do not rule out subtle quantitative changes in TGF-β expression or even major changes in bioavailability, however.

In contrast, another immunohistochemical analysis of 120 benign and malignant breast tissue samples showed that 38% of invasive carcinomas (82 cases studied) had elevated levels of TGF-β expression in the epithelial component when compared with normal tissue (10 cases) or with carcinoma in situ (4 cases) [67]. The relatively small number of normal samples and the difficulties of quantitating immunohistochemical data make this comparison rather difficult to evaluate. There appeared to be a weak positive correlation between progesterone receptor status and TGF-β expression, and TGF-β-positive tumors were associated with a better prognosis for relapse-free interval (followed over only 2 years) than tumors with no TGF-β staining, although there was no difference in the survival rate. Similarly, a survey of TGF-β1 mRNA expression in 52 malignant breast biopsies and 15 nonmalignant ones showed that a significantly higher fraction of the malignant samples (76%) showed higher TGF-β1 mRNA levels than the nonmalignant ones (38%) [68]. Interpretation of these data is confounded somewhat by the variable cellularity of the samples, although no correlation was observed between TGF-β levels and lymphocytic infiltration.

In vitro, little difference in TGF-β expression between normal and malignant cells is seen at the mRNA level with established mammary

carcinoma lines [69]. Normal HMEC and mammary tumor cell lines showed roughly equivalent amounts of TGF-β1 mRNA, although T47D cells appear to be an exception, with very low basal expression of TGF-β1 mRNA [69–72]. No correlation was observed between TGF-β1 mRNA levels and estrogen receptor status of the tumor line [72]. Less work has been done with TGF-β2 and TGF-β3, but some cell lines have been shown to express the message for these isoforms as well. Thus T47D expressed mRNA for all three subtypes, ZR-75-1 expressed TGF-β1 and TGF-β3, while MDA-MB-231 expressed TGF-β1 and TGF-β2 [71]. At the protein level, all lines tested secreted TGF-βs, although estrogen receptor-negative (ER−) cell lines MDA-MB-231, MDA 330, Hs578T, and BT20) secreted more TGF-β than did ER-positive (ER+) lines (MCF-7, T47D, and ZR 75-1) [73,74]. This difference was not due to the suppression of TGF-β secretion in ER+ lines by estrogen, since experiments were performed in phenol-red-free medium, using serum stripped of endogenous steroids. It may reflect some real mechanistic link between the ER status and TGF-β production or an artifactual difference in the response of ER− and ER+ cells to tissue culture pressures. The latter seems more likely, since the two immunohistochemical studies of breast carcinoma samples indicated no correlation between TGF-β expression and ER status [53,67]. A series of immortal, partially transformed, and fully tumorigenic cell lines generated in vitro by carcinogen treatment and oncogene transformation of normal human mammary epithelial cells showed essentially no change in secreted TGF-β levels when compared with the normal cells [57].

**4.2.2. Alterations in growth response to TGF-β.** There are conflicting reports in the literature on the responsiveness of mammary tumor-derived cells to growth inhibition by TGF-βs in vitro. Some of these differences may be due to the effects of culture conditions of TGF-β responses. For example, the inhibitory effect of TGF-β on a number of tumor cell lines in vitro, including MCF-7, has been shown to be dependent on the presence of polyunsaturated fatty acids (PUFAs) and was reversed by antioxidants or selenite [75 and unpublished data cited therein]. Furthermore, maximal growth inhibition required the presence of retinoids under some conditions. In contrast, growth inhibition of nontransformed epithelial and fibroblastic cells was insensitive to PUFAs. Given the highly variable levels of PUFAs, antioxidants, and retinoids in different batches of serum, the author proposed that these observations may explain some of the variability in the responsiveness of malignant cells to TGF-βs in culture [75]. Other differences in the response may be due to genetic drift or epigenetic changes occurring in the various malignant lines during long-term culture in different laboratories.

The data for the effects of TGF-βs on the growth of mammary tumor cell lines are summarized in Table 1. The controversy mainly centers around whether ER status predicts responsiveness to TGF-β. While it is clear that

*Table 1.* Effects of TGF-βs on the growth of human breast tumor cell lines

| Cell | ER status | Effect of TGF-β on growth | Growth conditions: Anchorage independent | Growth conditions: Anchorage dependent | Serum | Ref. |
|---|---|---|---|---|---|---|
| MCF-7 | ER+ | None | | + | + | 76 |
| | | None | | + | − | 77 |
| | | None | + | + | + | 74 |
| | | None | + | | + | 78 |
| | | Inhibition (weak) | + | | + | 78 |
| | | Inhibition | | + | + | 71 |
| | | Inhibition | + | | + | 79 |
| | | Inhibition | + | | + | 16 |
| | | Inhibition | | + | + | 80 |
| T47D | ER+ | None | + | + | + | 74 |
| | | None | + | | + | 71 |
| | | None | + | | + | 78 |
| | | Inhibition (weak) | + | | − | 78 |
| | | Stimulation | | + | + | 72 |
| ZR75-1 | ER+ | None | + | + | + | 74 |
| | | Inhibition (weak) | + | | + | 78 |
| | | Inhibition | | + | + | 81 |
| BT20 | ER− | Stimulation | | + | +/− | 82 |
| | | Inhibition | + | | + | 82 |
| | | Inhibition | + | + | + | 74 |
| HBL 100 | ER− | None | | + | + | 82 |
| | | Inhibition | | + | − | 82 |
| | | Inhibition (weak) | + | | + | 82 |
| Hs578T | ER− | Inhibition | + | | + | 78 |
| | | Inhibition | + | + | + | 74 |
| | | Inhibition | + | | + | 83 |
| | | Inhibition | | + | − | 83 |
| MDA-MB-231 | ER− | Stimulation | | + | + | 82 |
| | | Inhibition | | + | − | 82 |
| | | Inhibition | + | | + | 82 |
| | | Inhibition | + | + | + | 78 |
| | | Inhibition | + | + | + | 74 |
| | | Inhibition | + | + | + | 79 |
| | | Inhibition | + | | + | 83 |
| | | Inhibition | | + | − | 83 |
| MDA-330 | ER− | Inhibition (weak) | + | + | + | 74 |
| MDA-MB-435 | ER− | Inhibition | | + | + | 84 |
| MDA-MB-468 | ER− | Inhibition | + | | + | 78 |
| SK-BR-3 | ER− | None | | + | +/− | 82 |
| | | Inhibition | + | | + | 82 |
| | | Inhibition | + | | + | 78 |

In most of these studies, TGF-β1 was used. However, in one study, both TGF-β1 and TGF-β2 were found to be equivalently active on all tumor lines tested [78]. ER = estrogen receptor.

no absolute distinction can be made along those lines, it seems that ER− lines may be somewhat more responsive to growth inhibition by TGF-β than ER+ lines. Lack of response in some ER+ lines correlated with loss of the TGF-β receptors [74], a relatively rare event in other tumor types. How-

ever, HMECs transformed in vitro retained responsiveness to TGF-βs, even in the highly tumorigenic cells generated by transformation with multiple oncogenes [57]. Furthermore, anti-TGF-β antibodies stimulated the growth of MDA-231 and Hs578T mammary carcinoma cells in vitro, indicating that these cells have a fully functional negative autocrine loop [83]. These last results are particularly significant since the cells were shown to secrete TGF-β predominantly (>99%) in the latent form [83]. Thus the stimulatory effect of the antibodies suggests these particular tumor cells are probably capable of activating the latent TGF-β they produce. It will be important to determine whether this is true for other tumor lines too.

In contrast to these results obtained on cell lines that have been maintained long-term in culture, a study using primary cultures of cells that grew out from a fragment of malignant breast tissue derived from a mastectomy sample demonstrated that TGF-β had no inhibitory effect on the malignant cells, despite inhibiting the growth of cells derived from nonmalignant regions of the same sample [56]. The results of this type of study may be more meaningful than results obtained on cells after extended periods in culture. However, more samples will have to be analyzed to determine whether loss of inhibition by TGF-β is a general feature of freshly prepared breast tumor cells. Other evidence suggests that long-term culture may indeed restore a response that is not expressed in vivo. Thus, the MDA-MB-231 mammary carcinoma line was potently inhibited by TGF-β1 and TGF-β2 in vitro, but chronic administration of TGF-β1 had no effect on the growth of the tumor in nude mice [85].

In summary, it appears that tumorigenesis in the mammary gland is certainly not associated with any decrease in TGF-β expression in the epithelial component, nor any major change in the pattern of isoforms expressed. Rather, TGF-β expression appears to remain unchanged or even to be slightly elevated in tumor tissue, particularly in tumors showing a strong desmoplastic response. This increase in TGF-β expression could represent a homeostatic response to the increased cellular proliferation, or it may represent the selection of variant cells expressing higher TGF-β levels, since this may confer an advantage for invasion and metastasis at late stages of tumor development (see Section 5.5).

Furthermore, the data with established breast tumor cell lines in vitro suggest that the malignant phenotype is not strictly coupled to the loss of growth inhibition by TGF-β. However, the dependence of the response to TGF-β on the local cellular microenvironment may make it impossible to determine in vitro whether a tumor cell is refractory to growth inhibition by TGF-β in vivo. In addition, the more complex question of whether tumor stromal cells lose responsiveness to TGF-β in such a way as to reduce paracrine inhibitory effects of these cells on the epithelium has not been addressed. It therefore remains an open question as to whether the loss of the growth-inhibitory response to TGF-β may be important in the genesis of mammary carcinomas in vivo.

*Table 2.* Summary of the effects of TGF-βs on interstitial matrix and basement membrane components

| Interstitial matrix | Basement Membrane |
|---|---|
| Effects of TGF-βs on individual components | |
| A. Increased matrix protein synthesis<br>↑ fibronectin<br>↑ collagens I, III, V<br>↑ proteoglycans<br>↑ vitronectin<br>↑ thrombospondin | A. Increased matrix degradation<br>↑ type IV procollagenase<br>↑ activation of procollagenase<br>↓ inhibitor (TIMP-2) |
| B. Increased matrix deposition<br>↑ synthesis and processing of integrins (matrix binding proteins) | |
| C. Decreased matrix degradation<br>↓ synthesis of proteases; interstitial collagenases, plasminogen activator, plasmin<br>↑ synthesis of protease inhibitors; plasminogen activator inhibitor, TIMP-1 | |
| Predicted net effect | |
| —Matrix stabilization—<br>Confirmed in vitro and in vivo | —Matrix degradation—<br>Not yet determined |

## 5. TGF-β effects on stromal elements

Development of a malignant tumor is not just dependent on increased cellular proliferation, but involves a complex series of changes that allow the malignant cell to escape the confines of the surrounding stroma, to evade the immune surveillance system, and to colonize successfully distant organs. The potential involvement of TGF-βs in these processes is considered below.

### *5.1. Extracellular matrix*

Many of the biological activities of the TGF-βs appear to be mediated through effects on extracellular matrix [for a review, see 5]. In general, TGF-βs promote and stabilize the deposition of interstitial matrix by multiple mechanisms (Table 2). The net effect of TGF-β is to increase interstitial matrix expression, and there is considerable evidence that this occurs in vivo [50,86].

In infiltrating ductal and lobular breast carcinomas there was particularly strong extracellular staining for TGF-β1 in the stromal matrix of those tumors that showed an intense desmoplastic reaction (Figure 8D) [53]. Although strong staining for TGF-β was observed in the tumor cells of medullary breast carcinomas, in this case no extracellular TGF-β was found in the stroma, which consisted predominantly of inflammatory cells, and no

fibrosis was observed. The results are consistent with the elevated levels of TGF-β in the stroma of the desmoplastic tumors driving the increased matrix deposition. The extracellular TGF-β may be secreted by the tumor cells themselves, or the malignant epithelium in these tumors may be inducing the stromal cells to secrete more TGF-β, which then stimulates matrix deposition in an autocrine fashion. However, the alternative explanation that the extensive desmoplastic matrix may simply bind and sequester TGF-β in unusually high quantities cannot be discounted. Indeed, it is possible that TGF-β sequestration by the desmoplastic matrix may actually interrupt a negative autocrine or paracrine control path. Medullary carcinomas have a better clinical prognosis than the ductal and lobular carcinomas, and show no such matrix binding.

In contrast to the matrix-stabilizing effects of TGF-β on interstitial matrix components, TGF-βs may actually promote degradation of the basement-membrane matrix components. Recent work has shown that TGF-βs specifically increase the levels of the $M_r$ 72,000 type IV collagenase activity in a variety of tumorigenic human cell lines in which the interstitial collagenase was regulated in the opposite direction [87]. Increase in the activity of the type IV collagenase appeared to occur at two levels. TGF-β caused an increase in mRNA levels for type IV collagenase, resulting in an increased expression of the 72-kDa proenzyme, and further enhanced the cellular processing of the latent proenzyme to active lower molecular weight forms in some cell types. Furthermore, TGF-β specifically decreased the expression of a novel inhibitor, TIMP-2, which inhibits the type IV collagenase [88]. This suggests that elevated TGF-β expression might facilitate invasion through the basement membrane in vivo.

Consistent with this hypothesis, pretreatment of a mammary adenocarcinoma clone MTLn3 with TGF-β caused a two- to threefold increase in the number of lung surface metastases seen when cells were inoculated into syngeneic rats [89]. Increased metastatic potential was associated with increased ability of the cells to extravasate, as determined by their ability to invade through an artificial basement membrane in vitro. This property, in turn, correlated with increases in type IV collagenolytic and heparanolytic activities, and was not due to enhanced association with the basement membrane. Similarly, in a malignant mouse fibrosarcoma model, metastatic potential correlated with an increased expression of active TGF-β compared with the nonmetastatic parent [90].

However, experiments on human mammary adenocarcinoma lines have indicated more complex effects of TGF-β on invasiveness through basement membranes in vitro [91]. In these experiments, TGF-β had no effect on the migration or invasiveness of MCF-7, T47D, or MDA-MB-468 cells; inhibited the invasiveness but not the migration of MDA-MB-436 cells; and inhibited both invasiveness and migration of the more malignant, hormone-independent Hs578T and MDA-MB-231 cells. The invasiveness of early-passage MCF-7 cells appeared to be stimulated by TGF-β. Similarly, in

the oncogene-transformed A1N4 series of breast epithelial lines, TGF-β inhibited the invasiveness of only the most aggressive A1N4-TH cells, while inhibiting the migration of all the cell lines (E.W. Thompson, personal communication). Interestingly, TGF-β increased levels of the type IV procollagenase in MDA-MB-436 and A1N4-TH cells, despite inhibiting their invasiveness (E.W. Thompson and P.D. Brown, personal communication). This suggests TGF-β has pleiotropic effects on multiple components of the invasive phenotype and that the positive effects on basement membrane degrading enzymes may not always dominate.

### *5.2. Stromal-epithelial interactions*

The intimate interdependence of parenchyma and stroma in normal growth and development of the breast [92] suggests that lesions in either element might disrupt normal tissue homeostasis. Experimentally it will be much harder to identify defects in paracrine rather than autocrine signaling paths involving TGF-β because of the need for complicated coculture systems. However, this is clearly an important area that merits further study.

An interesting paracrine loop involving TGF-β and tenascin has been identified in cocultures of breast cancer and breast fibroblast cells. Tenascin is an extracellular matrix glycoprotein that plays an important role in cell–cell interactions and cell migration during mammary organogenesis. It is consistently overexpressed in the stroma of malignant breast tumors [93] and, experimentally, tumor epithelium has been shown to induce the surrounding stroma to lay down tenascin-containing matrix in vivo [94]. Experiments in vitro demonstrated that MCF-7 mammary carcinoma cells secreted TGF-β, which stimulated the production of tenascin by fibroblasts [95]. The tumor cells reacted to exogenous tenascin by losing cell–cell and cell–substrate contact and proliferating more rapidly. Thus a deleterious paracrine loop was established in which TGF-β produced by the malignant breast cells caused increased secretion of tenascin by neighboring fibroblasts. This in turn increased malignant cell growth and detachment, which would be predicted to favor the metastatic phenotype. In a similar vein, it will be interesting to determine whether TGF-β has any effect on the expression of cadherins, the family of receptors that mediate selective cellular adhesion of particular cell types [96]. Downregulation of the epithelial cadherin, E-cadherin, in tumor cells is associated with a propensity to metastasize, and experimental addition of anticadherin antibodies promotes the invasion of normal tissues by tumor cells [96].

There is evidence that fibroblasts from patients with breast cancer, or from disease-free individuals with a familial predisposition to breast cancer, have an abnormal phenotype, manifested by an unusually high migratory capacity [97,98]. This is due to expression by these cells of a 'migration stimulating factor' (MSF), a factor that is normally secreted by fetal fibroblasts [98,99]. MSF is thought to act at least in part through a stimulation of

hyaluronic acid synthesis [100]. TGF-β1 and TGF-β2 antagonized the action of MSF on migration and hyaluronic acid synthesis in vitro (S. Schor, personal communication). In this case, therefore, TGF-β produced by the epithelium might neutralize an underlying stromal defect. Along the same lines, it will be interesting to see whether TGF-β has any effect on the expression or activity of stromelysin-3, a novel metalloproteinase that is expressed in all invasive breast cancers, with expression restricted to the stromal cells immediately surrounding the neoplastic cells of the invasive, but not the in situ, component of breast carcinomas [101].

### *5.3. Effects on the immune system*

TGF-βs have potent effects on the functioning of many cells of the immune system. In particular, they suppress the generation of specific cytotoxic T-lymphocytes (CTLs) and the activation of natural killer (NK) cells [102]. Interestingly, CTL and NK cells appear to respond equivalently to active and latent TGF-β, suggesting they can efficiently activate latent TGF-β [103]. Stable transfection of a highly immunogenic murine fibrosarcoma line with the TGF-β1 gene prevented the malignant cells from eliciting a CTL response in vitro, or in vivo [104]. This appeared to be a direct suppressive effect of the TGF-β on the effector cells, since there was no change in the expression of tumor antigens or major histocompatability complex components in the transfected cell line. TGF-β producing tumors grew pro-

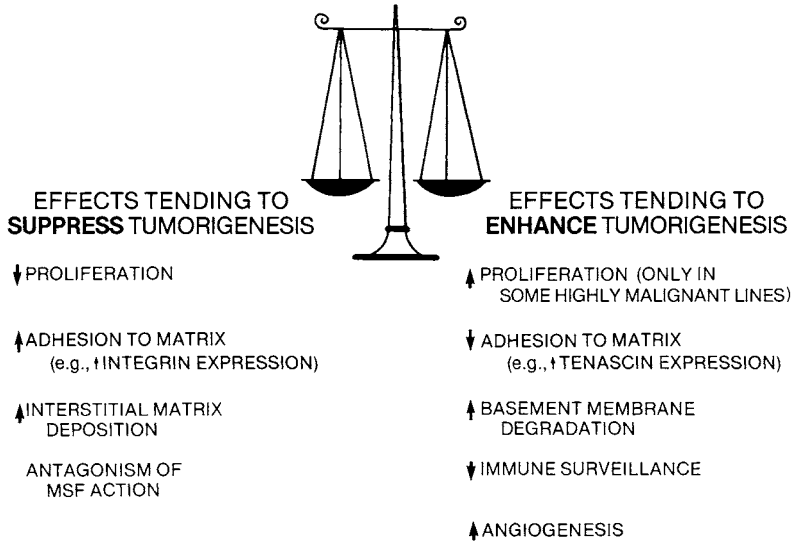


*Figure 9.* Activities of TGF-β that may affect tumorigenesis.

*Table 3.* Effects of various steroids on the expression of TGF-βs by human breast tumor cell lines

| Cell | Steroid/analog | Effect on TGF-β expression | | TGF-β subtype | Ref. |
|---|---|---|---|---|---|
| | | Protein | mRNA | | |
| BT-20 | Tamoxifen | None | n.d. | β1 | 10 |
| | Gestodene | Increase (3x) | n.d. | β1 | 10 |
| MCF-7 | $E_2$ | Decrease | n.d. | n.d. | 73 |
| MCF-7[a] | $E_2$ | n.d. | Decrease | β1 | 109 |
| MCF-7 | $E_2$ | n.d. | None | β1 | 71 |
| | Tamoxifen | n.d. | None | β1 | 71 |
| MCF-7 | $E_2$ | Decrease (2x) | n.d. | n.d. | 79 |
| | LY117018 | Increase (27x) | None | n.d. | 79 |
| | Tamoxifen | Increase (5x) | n.d. | n.d. | 79 |
| | 4-hydroxy-tamoxifen | Increase (18x) | n.d. | n.d. | 79 |
| | Dexamethasone | Increase (2x) | n.d. | n.d. | 79 |
| MCF-7 | Tamoxifen | Increase (4x) | n.d. | β1 | 10 |
| | Gestodene | Increase (10x) | n.d. | β1 | 10 |
| MDA-MD-231 | $E_2$ | n.d. | None | β1,β2 | 71 |
| MDA-MB-231 | Tamoxifen | None | n.d. | β1 | 10 |
| | Gestodene | Increase (6x) | n.d. | β1 | 10 |
| T47D | MPA | n.d. | Decrease | β1 | 70 |
| T47D | $E_2$ | Decrease | n.d. | n.d. | 71 |
| | $E_2$ | n.d. | Decrease | β2,β3 | 71 |
| | $E_2$ | n.d. | None | β1 | 71 |
| | Tamoxifen | n.d. | None | β1–3 | 71 |
| T47D[b] | $E_2$ | n.d. | None | β1 | 110 |
| | $E_2$ | n.d. | Decrease | β2 | 110 |
| | Tamoxifen | n.d. | None | β1 | 110 |
| | Tamoxifen | n.d. | Increase | β2 | 110 |
| T47D | Tamoxifen | Increase (32x) | None | β1 | 10 |
| | Toremifene | Increase (24x) | n.d. | β1 | 10 |
| | Gestodene | Increase (94x) | Increase | β1 | 10 |
| T47D-A18 | $E_2$ | n.d. | Increase | β1 | 111 |
| | $E_2$ | n.d. | Decrease | β2 | 111 |
| ZR-75-1 | $E_2$ | n.d. | Decrease | β3 | 71 |
| | $E_2$ | n.d. | None | β1 | 71 |
| | Tamoxifen | n.d. | None | β1,β3 | 71 |

n.d. = not determined; $E_2$ = estradiol; MPA = medroxyprogesterone acetate. Tamoxifen, 4-hydroxytamoxifen, LY117018 and toremifene are all antiestrogens. Gestodene is a synthetic progestin.

[a] This experiment was performed in vivo, using a xenograft tumor system.

[b] These cells had been adapted for growth in the absence of steroids over an extended period of time.

gressively in transiently immunosuppressed mice, although they did not grow progressively in normal recipients, presumably because a fraction of the CTL cells specific for the tumor line escaped initial suppression by TGF-β. Thus tumors producing TGF-β in large quantities may be able to evade immunosurveillance, at least in situations where the immune system is somewhat compromised.

### *5.4. Angiogenesis*

Although TGF-β is inhibitory for the growth of endothelial cells in vitro, in vivo it appears to enhance angiogenesis. Indeed, during embryogenesis there is a strong correlation of expression of TGF-β1 with areas of extensive neovascularization [105], and TGF-β1 injected into the nape of the neck of newborn mice evokes the production of granulation tissue with characteristic new blood vessel formation [86]. This effect may be indirect due to the recruitment of monocytes and their activation to secrete angiogenic factors, such as tumor necrosis factor-alpha [106]. Thus overproduction of TGF-β by a tumor may promote neovascularization of the tumor by similar mechanisms.

### *5.5. Implications of tumor development, invasion, and metastasis*

While the induction of interstitial matrix components by TGF-βs might decrease the propensity of a tumor to metastasize by physically confining it, other properties of the TGF-βs are likely to promote invasion and metastasis. The induction of enzymes that degrade the basement membrane, the induction of components such as tenascin that promote cell detachment and motility, inhibitory effects on the immune surveillance system, and the promotion of neovascularization would all tend to promote metastasis. All these effects are examples of aberrant paracrine communication between epithelium and stroma. These various effects are summarized in Figure 9. Currently it is not clear which of these effects will dominate at what stage in tumor development. Since the metastatic phenotype arises late in tumorigenesis, elevated TGF-β expression at early stages may tend to have an antitumor effect through increased growth inhibition, while elevated TGF-β at later stages might be deleterious, tending to promote successful invasion and metastasis. Consistent with this, in a malignant mouse fibrosarcoma model, and in benign and malignant rat liver cell lines, metastatic potential was correlated with an increased expression of active TGF-β [90]. Furthermore, TGF-β may actually be growth stimulatory rather than inhibitory in some highly malignant carcinoma cell lines [72,82,107].

## 6. Regulation of TGF-βs by members of the steroid hormone superfamily

### *6.1. Effects of steroids on TGF-β production by breast cancer cells*

A major conceptual advance in understanding the growth regulation of hormone-dependent tumors came with the demonstration that estrogen stimulates the growth of hormone-dependent breast tumor cells in part by the induction of autocrine growth-stimulatory factors such as TGF-α [108]. Conversely, it was proposed that estrogen might suppress levels of endog-

enous growth inhibitors and that estrogen withdrawal or treatment with antiestrogens might stimulate the production of these inhibitors [79].

Early experiments showed that estrogen treatment caused an approximately twofold decrease in TGF-β protein expression in MCF-7 cells under steroid-free conditions [73,79], whereas the antiestrogens tamoxifen, LY117018, and 4-hydroxy tamoxifen caused an 8- to 27-fold induction of TGF-β protein [79]. The induction of TGF-β was mediated through the estrogen receptor, since no induction was seen in ER− lines, and the effect could be antagonized by increasing concentrations of estrogen. In addition to increasing the overall secretion of TGF-β, antiestrogens also increased the fraction of the TGF-β that was in the biologically active form, from ~5% of the total in control cells to 18% in treated cells. Anti-TGF-β antiserum reversed most of the inhibitory effect of conditioned medium from MCF-7 cells treated with antiestrogens on the ER− breast cancer line MDA-MB-231, suggesting that TGF-β was the principal growth-inhibitory activity made by these cells in response to antiestrogens. Furthermore, the data indicated that TGF-β induced in ER+ cells by antiestrogen treatment could have a paracrine-inhibitory effect on neighboring ER− cells, as well as direct effects on ER+ cells.

Subsequently numerous other groups have examined the effects of estrogen and antiestrogens on TGF-β production by breast cancer cell lines. The results have tended to be complex and are summarized in Table 3. The regulation of TGF-β by estrogen and antiestrogens appears to be critically dependent on the culture history of the cell and the culture conditions of the experiment. For instance, long-term withdrawal of steroids from ER+ cell lines in culture can result in changes of baseline expression of the various TGF-β subtypes, as well as changes in the response to estrogens and antiestrogens [110]. Thus long-term estrogen withdrawal of ZR-75-1 cells resulted in a decrease in TGF-β1 mRNA, while long-term steroid deprivation of T47D cells caused an increase in the basal expression of both TGF-β1 and TGF-β2 mRNAs. These adaptive changes in long-term culture can be variously the result of epigenetic or genetic alterations [72].

Since regulation of TGF-β1 by antiestrogens appears to involve post-transcriptional mechanisms in vitro [10,11,79], studies that have only looked at TGF-β1 mRNA levels could have overlooked a post-transcriptional induction. Where protein levels were determined, there is fairly general agreement that antiestrogens can induce TGF-β protein in ER+ cell lines (see Table 3). However, neutralizing TGF-β antibodies only partially reversed the growth-inhibitory effects of antiestrogens in some systems, suggesting the involvement of additional growth inhibitors or inhibitory pathways [112].

Modulation of TGF-β expression by steroids can also be seen in vivo. Estradiol ($E_2$) withdrawal from MCF-7 cells innoculated in nude mice was associated with a threefold increase in mRNA for TGF-β1 in the tumor [18], and conversely, $E_2$ administration to MCF-7 xenografts in thymectomized

irradiated mice caused a decrease in TGF-β1 mRNA levels [109]. The effects of antiestrogens on TGF-β expression by breast cancer cells in vivo have not yet been investigated. Interestingly, some MCF-7 variants respond to $E_2$ ablation in vivo by increased programmed cell death or apoptosis [18]. Apoptosis is associated with an increase in TGF-β1 mRNA in the tumor, and TGF-β has been shown to induce apoptosis in prostatic glandular cells in organ culture [19]. It will be important to determine how cytotoxic paths may be coupled to TGF-β action in other breast cancer lines that do not show this response.

### *6.2. Induction of TGF-β in stromal fibroblasts by antiestrogens*

There is evidence that antiestogens may be able to induce TGF-β secretion from fibroblasts in the apparent absence of a functional ER [11]. Human fetal fibroblasts were chosen to model the fetal-like phenotype of breast tumor stromal fibroblasts [98]. Treatment of these cells with antiestrogens caused a 5–20× increase in secreted TGF-β1 [11]. As with the breast cancer cells, TGF-β induction was post-transcriptional, involving primarily TGF-β1, and a large fraction (70–100%) of the secreted TGF-β was in the biologically active form. The effect was not reversible by estrogen, suggesting that the classical ER was not involved, and additional experiments indicated that this receptor was absent or only expressed at very low levels. A steroidal pure antiestrogen ICI 164,384 was also effective in inducing TGF-β. Since this antiestrogen does not bind to the cytoplasmic 'antiestrogen binding sites,' this suggested that antiestrogens were inducing TGF-β through the agency of some novel antiestrogen binding protein. The demonstration that antiestrogens can induce TGF-β secretion by stromal fibroblasts provides a possible rationalization for the clinical observation that women with ER− breast tumors show some response to antiestrogen treatment [113]. It also suggests the feasibility of making the tumor stroma a target for therapeutic intervention.

### *6.3. Induction of TGF-β in breast cancer cells by a novel synthetic progestin*

A novel synthetic progestin, gestodene, has been identified that is a more potent inhibitor of breast cancer cell growth in vitro than the antiestrogens [10]. Binding studies have identified a unique intracellular binding protein for gestodene that differs from the classical progesterone receptor and appears to mediate the growth-inhibitory effects [10,114]. This gestodene binding protein shows a very specific tissue distribution; it is only expressed in malignant breast tissue and breast tumor cell lines, and is absent from other malignancies and from normal tissue [115]. Acting through this novel binding protein, gestodene caused a dramatic (up to 90×) induction of active TGF-β1 and, to a lesser extent, TGF-β2 in breast cancer cell lines [10]. The extent of induction was proportional to the levels of gestodene

binding protein expressed and classical progestins had no effect, confirming that the progesterone receptor was not involved. As with the antiestrogens, the induction of TGF-β1 was largely post-transcriptional, and the TGF-β secreted was in the biologically active form. Furthermore, the growth-inhibitory effect of gestodene was partially reversed by anti-TGF-β antibodies, suggesting an important role for TGF-β in mediating the growth inhibition. Additional analogs of gestodene have now been synthesized that are more potent in inhibiting the growth of breast tumor lines, and their effects on TGF-β production are being analyzed (our unpublished data). The very specific expression of the gestodene receptor only in malignant breast tissue offers a unique opportunity for preventive or therapeutic intervention (see Section 6.5).

It seems likely that the growth-inhibitory actions of other unrelated agents on breast cancer cells in vitro may also be mediated by the induction of TGF-βs. For example, the inhibition of ZR-75 breast carcinoma cells in vitro by alpha-interferon can be partially reversed by anti-TGF-β antibodies [81]. Conversely polyamines, which stimulate the growth of MCF-7 cells in vitro, decreased the secretion of TGF-β, and inhibitors of polyamine synthesis had the reverse effect [77]. Possibly the TGF-βs represent the dominant and most proximal signaling channel for growth inhibition in the breast, and this common pathway is therefore activated by a variety of structurally distinct growth-inhibitory agents.

### *6.4. Regulation of TGF-βs by other steroids in nonbreast systems*

Other steroids that may be relevant to the breast cancer system regulate expression of different TGF-β subtypes in a variety of systems. Retinoids appear to play a particularly important role in regulating TGF-β expression in epithelial tissues in vitro and in vivo [116]. Although TGF-β1 and -β3 expression were regulated to some extent, the most dramatic effects of retinoids were seen on the expression of TGF-β2. In vivo, the direction of the effect depended on the nature of the target epithelium. Retinoic acid induced TGF-β2 expression in dermal, colonic, and lung epithelia but inhibited it in vaginal epithelium. Given the overlapping spectrum of activities of retinoids and TGF-βs in regulating growth and differentiation, it seems likely that some of the effects of retinoids in vivo may be mediated by local induction of TGF-βs. The two may also synergize or interact at other levels. Thus in MCF-7 variants that are refractory to direct inhibition by TGF-β, TGF-β strongly potentiated the growth-inhibitory effects of retinoic acid [117]. Dihydroxyvitamin $D_3$ has also been shown to regulate TGF-β expression in some cells [118].

Mechanistically, regulation of expression of TGF-β subtypes by steroid hormone family members can occur at multiple levels. Frequently regulation is post-transcriptional; retinoic acid induction of TGF-β2 in keratinocytes probably involves changes in mRNA stability [9], whereas regulation

of TGF-β1 expression by antiestrogens and progestins involves changes in translatability of preexisting mRNA, or downstream effects on post-translational stability, processing and modification, assembly, or secretion [10,11,79]. Since the three TGF-β subtypes differ markedly in the structures of their promoters, 5′ and 3′-untranslated regions, and somewhat in the sequence of the precursor pro region, there is ample potential for differential regulation of the subtypes by steroids at any of these levels. It is also particularly noteworthy that TGF-β recovered from the conditioned medium of cells treated with steroids or retinoids is frequently in the biologically active, rather than the more common latent form [9–11,79], suggesting possible effects of these agents on the activating mechanism. Experiments with other systems have confirmed that steroids and retinoids may have additional effects on TGF-β regulatory loops. For example, treatment of osteoclasts with retinoic acid enhances their ability to activate exogenous latent TGF-β [119], and retinoids sensitize certain cell types to growth inhibition by TGF-β by mechanisms involving events downstream of receptor binding [120,121]. The interface between the biology of the TGF-βs and that of the steroid hormone superfamily has only begun to be explored but will doubtless be extensive.

## 7. Implications for chemoprevention and chemotherapy of breast cancer

Increased cell proliferation plays a key role in the pathogenesis of all human cancers [2]. It follows logically that growth inhibitors should delay or prevent the development of a malignant metastatic tumor and possibly be effective in the treatment of established tumors. The potent growth-inhibitory effects of TGF-βs on mammary epithelial cells make these peptides interesting candidates for novel preventive or therapeutic modalities.

The results of early experiments attempting to use exogenous TGF-βs to inhibit the growth of established tumors have been discouraging. The human breast cancer line MDA-MB-231 is potently inhibited by TGF-β1 and -β2 in vitro [78]. However, MDA-MB-231 breast tumors in nude mice are only transiently growth inhibited by TGF-β administered by a variety of routes [85]. Furthermore, systemic administration of high doses of active TGF-β was associated with disseminated organ fibrosis and cachexia. Similar negative results in vivo have been obtained with the TGF-β-sensitive human lung adenocarcinoma line A549 [122], although other investigators did observe an inhibition of tumor growth with this cell line when TGF-β was injected peritumorally [123].

The lack of response of these tumors to TGF-β in vivo may be due to a number of factors. First, it may reflect a poor understanding of the pharmacokinetics of TGF-β. Secondly, as noted in Section 5.5, TGF-β has biological activities that might actually enhance the growth of cell lines derived from highly malignant tumors in the complex in vivo environment.

Thirdly, the dependence of the TGF-β growth-inhibitory effects in vitro and in vivo on microenvironmental influences, such as the growth substratum, may indicate that, in addition to delivering TGF-β to the tumor, it may be necessary to intervene in such a way as to make the tumor cells more sensitive to TGF-β effects in vivo.

The results suggest that we will have to be more sophisticated in our approach to this problem. The undesirable effects of TGF-β on tumors that have already progressed a long way down the path to aggressive malignancy, and the well-known heterogeneity of such tumors, indicate that TGF-βs may be most usefully employed early in breast disease as preventive rather than therapeutic agents. The delivery problem and the unwanted side effects associated with the systemic administration of active TGF-β could be cirumvented by using pharmacological agents to manipulate endogenous TGF-β levels in target premalignant or malignant tissues. As indicated above, members of the steroid hormone superfamily have considerable potential in this regard, since they induce the production of biologically active TGF-β in a variety of target tissues. Furthermore, if proliferation occurs in regions of the epithelium that are not inhibited by TGF-β in vivo, such as the lobular epithelium, it may be necessary to employ a second pharmacological agent that will sensitize these cells to TGF-β, possibly through effects on the surrounding extracellular matrix. Retinoids enhance growth-inhibitory effects of TGF-β in a fibroblast system [114] and induce a growth-inhibitory effect in an otherwise unresponsive MCF-7 cell line [117], and polyunsaturated fatty acids sensitize certain tumor cell lines to growth inhibition by TGF-β [75].

A major advantage of using steroids or retinoids for targetted induction of TGF-βs lies in the fact that the induced TGF-β is mostly in the biologically active form. This means that it is likely to act very locally, and all surrounding cells with receptors, including any that may be incapable of activating latent TGF-β, are potential targets. In addition, the induction of TGF-βs by certain steroids can be exquisitely tissue specific. For example, gestodene only induces TGF-β production by malignant breast cells [10]. It should be noted that the malignant epithelium need not necessarily be the target for TGF-β induction. TGF-β induced in stromal elements of the tumor could have direct paracrine-inhibitory effects on the tumor cells or could alter stromal properties so as to make them less permissive for tumor development and metastasis. The autocrine and paracrine routes by which tamoxifen might inhibit breast tumor cell growth through induction of TGF-β are summarized in Figure 10.

Since different steroids induce different TGF-β subtypes, by combining different steroids it may be possible to induce multiple TGF-β subtypes in a given tissue for a stronger inhibitory effect. For example, retinoids and gestodene synergize in causing a far more extensive inhibition of breast cancer cell lines than is seen with either agent alone (our unpublished data). This synergistic action may be due in part to the induction of multiple TGF-

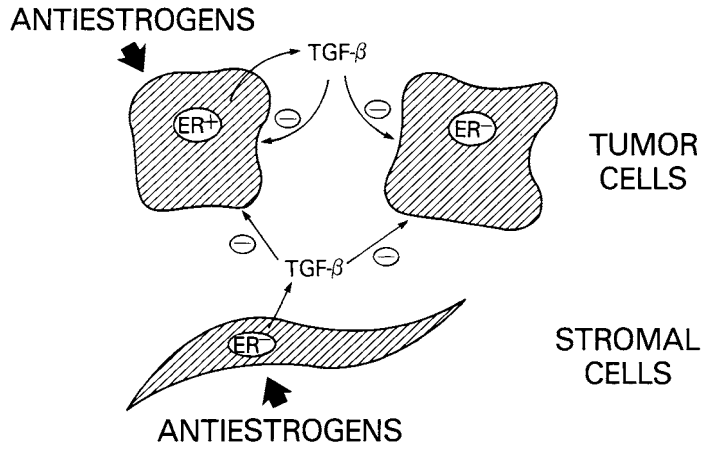


*Figure 10.* Possible routes for growth-inhibitory effects of antiestrogens on breast tumor cells, mediated by the induction of TGF-β. Antiestrogens can act directly on tumor cells expressing the estrogen receptor (ER+) to induce the secretion of active TGF-β. The TGF-β so produced can then inhibit the growth of the ER+ cells or neighboring ER− cells by autocrine and paracrine routes. In addition, antiestrogens can induce the secretion of active TGF-β from stromal fibroblasts by a novel mechanism that does not involve the estrogen receptor and can inhibit the growth of ER+ or ER− breast tumor cells in a paracrine fashion.

β subtypes or be due to mutually sensitizing phenomena, as mentioned above.

For people with familial risk factors predisposing them to certain tumors, one might elevate TGF-β levels chronically in the affected tissue. However, since this could give undesirable side effects, such as organ fibrosis, it would be preferable to induce TGF-β specifically and early only in the developing malignancy. If expression of the gestodene binding protein is an early event in malignant development, gestodene might be a candidate agent for the chemoprevention of breast cancer by this route. Gestodene acts as a classical progestin in the normal subject [124]. However, in a developing breast malignancy expressing the gestodene binding protein, gestodene may induce TGF-β and thereby slow down malignant progression (Figure 11). Since gestodene is already in use in Europe as a component of a combined oral contraceptive preparation, it will be interesting to see whether it does indeed protect against the development of breast cancer in the general population.

## 8. Future prospects

The identification and characterization of TGF-βs as endogenous inhibitors of mammary cell growth has increased our understanding of the delicate

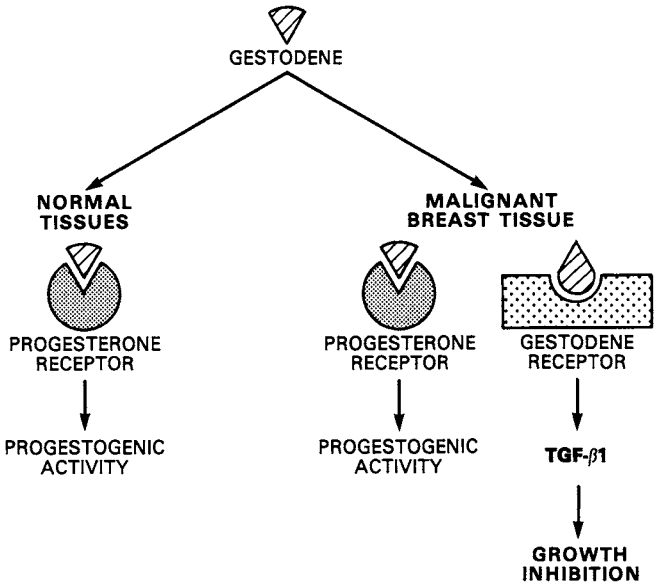


*Figure 11.* Proposed mechanism for gestodene acting as a potential chemopreventive agent. In the normal breast and other normal tissues, gestodene acts through the classical progesterone receptor, and has the progestogenic action that makes it useful as part of a combined oral contraceptive. In malignant breast tissue that expresses the novel gestodene receptor, gestodene would be predicted to induce the production of active TGF-β, resulting in local inhibition of surrounding premalignant or malignant cells.

balance of regulatory factors that goes into maintaining normal organ homeostasis. The data indicate complex roles for TGF-βs in mediating paracrine and autocrine cell communication in the normal mammary gland, with effects on cell growth and differentiation that vary in different regions of the gland and during different stages of development. Regardless of whether defects in regulatory interactions involving TGF-βs are important in the genesis of mammary tumors, it is clear that TGF-βs have potential in preventing this process. Indeed, the induction of TGF-βs appears to be a common mechanistic pathway that is activated by growth-inhibitory agents as structurally different as antiestrogens and interferons, suggesting that TGF-βs are fundamental components in the signaling language of growth inhibition in the breast.

Since members of the steroid hormone superfamily with proven efficacy in the prevention or treatment of breast cancers increase TGF-β synthesis in vitro, it may be possible to develop a new pharmacology for the chemoprevention of breast cancer based on the local induction of endogenous growth inhibitors, such as the TGF-βs, in or around the affected tissue. Complementary approaches might include the development of agents that

alter the bioavailability of endogenous TGF-βs in target tissues and agents that can enhance the cellular response to growth-inhibitory effects of TGF-βs. Indeed, optimal results are likely to be obtained with combinations of these strategies. Clinical trials have shown tamoxifen to be effective in preventing the recurrence of malignant breast disease [125], and animal studies indicate that combinations of antiestrogens and retinoids are more effective than either treatment alone [126].

The strong dependence of cellular responses to TGF-β on the cellular environment, and the potential importance of paracrine regulatory loops involving TGF-β between different cell types, underscores the need to focus on in vivo experiments. Studies in vitro have generally been more useful in explaining TGF-β effects observed in vivo than in predicting them, and studies in vivo are crucial to show which of the plethora of possible responses to TGF-β are actually manifested in the context of the appropriate cellular and matrix microenvironments. Similarly, a greater understanding of the ways in which the expression and bioavailability of the TGF-βs are controlled in vivo, and particularly elucidation of the molecular mechanisms by which TGF-βs exert their various biological effects, will be helpful in dissecting the useful activities from the less desirable ones. In particular it will be interesting to clarify and exploit possible mechanistic links between TGF-β and the process of programmed cell death. With such information in hand, the clinical goal of harnessing this potent growth inhibitor to good effect in the prevention or treatment breast cancer may then be achievable.

## Acknowledgments

We would like to thank the many people who generously communicated ideas and experimental results to us prior to publication. In particular we thank Drs. Charles Daniel, Gary Silberstein, Seth Schor, Peter Brown, Rik Thompson, and Gerhardt Zugmaier.

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PART III

# Oncogenes and stimulatory growth factors

# 7. Mechanisms involving an expanding *erb*B/EGF receptor family of tyrosine kinases in human neoplasia

Pier Paolo Di Fiore and Matthias H. Kraus

## 1. Dominant transforming genes and growth regulation

Compelling evidence for the involvement in the neoplastic process of molecules regulating normal cellular growth originates from observations demonstrating that the transduced oncogenes of certain acute transforming retroviruses represent activated versions of growth factors or growth factor receptors. When incorporated within the retroviral genome, such transduced sequences acquire the ability to induce neoplastic transformation. Analysis of viral oncogene products has revealed important insights into the function of their normal cellular counterparts, designated protooncogenes. For example, in AEV-induced neoplasia, the EGF receptor gene (c-*erb*B) is activated as an oncogene (v-*erb*B) upon amino-terminal truncation of the coding sequence combined with other modifications [1]. This has been observed for two independent transductions of the c-*erb*B locus into acute transforming retroviruses [2,3], as well as in c-*erb*B activation by promoter insertion during retroviral leukemogenesis [4]. The transforming gene of the feline sarcoma virus SM-FeSV represents another growth factor receptor tyrosine kinase, the CSF-1 receptor [5]. Oncogenic conversion by a retrovirus in this case (v-*fms*) involves more subtle structural alterations at the carboxyl terminus of the c-*fms* protooncogene [6].

Transfection analysis of high molecular weight genomic DNA in NIH3T3 fibroblasts has also led to the identification of structurally altered cellular oncogenes in spontaneous human neoplasia, as well as chemically induced experimental tumor model systems. The majority of oncogenes identified by this approach encompassed members of the *ras* gene family. In some instances, however, oncogenes other than *ras* genes have been detected. For example, transfection analysis of chemically induced rat neuroblastomas revealed the presence of a dominant transforming gene, termed *neu*, which exhibited an immunological relationship to the EGF receptor [7,8]. Oncogenic conversion of *neu* is due to a single point mutation in its transmembrane domain, which results in constitutive activation of its intrinsic tyrosine kinase function [9,10].

Whereas in experimental animal tumors evidence prevails for the ac-

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

tivation of growth factor receptors by structural alterations in the coding sequence, early observations of amplification of the complete epidermal growth factor receptor coding sequence in epithelial human neoplasia [11] linked this growth factor receptor to the most prevalent type of spontaneous human malignancies. This prompted our search for related molecules in this tyrosine kinase receptor family in order to investigate the role of mitogenic signaling pathways in the neoplastic process in vivo and in vitro. The findings supported the concept that growth-factor receptor alterations in spontaneous human neoplasia most commonly appear to involve gene amplification and/or overexpression of structurally intact transcripts and proteins. This, combined with functional in vitro characterization of such alterations, implies that enhancement of physiological growth signal transduction pathways may frequently exert a critical role in the development and progression of human malignancies.

## 2. The human *erb*B/EGFR gene family

Growth factor receptors are encoded by a family of ancestrally related genes that have segregated during evolution within the larger family of tyrosine kinases [12]. Receptor tyrosine kinases share a catalytic domain that represents the region of most extensive structural conservation and contains the determinants for the intrinsic enzymatic activity. Individual members in distinct subfamilies of receptor-like tyrosine kinases share closer structural and functional homology with each other than with other protein kinases [12]. This feature provided the basis for the identification of novel members in distinct receptor tyrosine kinase subfamilies. Quantitatively reduced hybridization stringency made possible the detection of subfamily-specific members in the absence of crosshybridization with molecules from distinct tyrosine kinase subfamilies [13].

Utilizing v-*erb*B as a probe, we have isolated and characterized two related receptor-like tyrosine kinases, designated *erb*B2 [14] and *erb*B3 [15], which share closer structural homology with the EGF receptor than with any other tyrosine kinase. While the initial identification of *erb*B2 was facilitated by its amplification in a primary human mammary carcinoma [14], the *erb*B3 gene was detected in normal human DNA [15]. Both *erb*B2 and *erb*B3 have been independently identified by others [16–19]. Independent isolations by different groups of the *erb*B2 gene gave rise to synonymous designations for this receptor tyrosine kinase, including c-*erb*B2 [17] and HER-2 [18]. Furthermore, sequence comparison and chromosomal mapping studies revealed that this molecule represents the human homologue of the rat *neu* gene [16]. *erb*B3 is also referred to as HER-3 [19].

In initial efforts to identify new genes of the *erb*B/EGF receptor subfamily, we employed hybridization conditions of moderate stringency under which protooncogenes related to other viral oncogenes of the tyrosine

kinase family did not hybridize. DNA prepared from a primary human mammary carcinoma, MAC117, showed a pattern of hybridization differing both from that observed with DNA of normal human placenta and the A431 squamous-cell carcinoma line with known amplification of the EGFR gene. In the mammary tumor, a single v-*erb*B-related-6kbp *Eco*RI restriction fragment appeared amplified distinct from EGF receptor-specific *Eco*RI fragments amplified in A431. These findings were consistent with the possibility that the MAC117 tumor contained an amplified DNA sequence related to, but distinct from the EGF receptor [14]. Molecular characterization of the 6-kbp fragment identified two exons with striking homology to both the human EGF receptor and v-*erb*B. A gene-specific probe detected a single 5-kb transcript in A431 cells. Under the stringent conditions of hybridization utilized, this probe did not detect any of the three RNA species recognized by EGF receptor complementary DNA. Thus, the gene, designated *erb*B2, represented a new functional gene, closely related to but distinct from the gene encoding the EGF receptor [14].

Subsequently, for the identification of *erb*B3 [15], normal human genomic DNA was cleaved with a variety of restriction endonucleases and was subjected to Southern blot analysis with v-*erb*B as the probe. Under the moderate stringency hybridization conditions employed for the identification of *erb*B2, three *Sac*I restriction fragments were detected. Two were identified as EGFR gene fragments by their amplification in MDA-MB468 cells known to contain EGFR gene amplification, and one as an *erb*B2-specific gene fragment due to its increased signal intensity in *erb*B2 amplified SK-BR-3 cells. When the hybridization stringency was reduced by 7°C, a new 9-kbp *Sac*I fragment was detected with v-*erb*B as a probe. This restriction fragment exhibited equal signal intensities in normal human thymus, MDA-MB468, and SK-BR-3 DNA. Taken together, these findings suggested the specific detection of a third v-*erb*B-related gene within the 9-kbp *Sac*I fragment [15].

Molecular cloning and characterization of three genomic exons within the 9-kbp DNA fragment revealed the highest identity scores of 64–67% to three regions that are contiguous in the predicted tyrosine kinase domains of v-*erb*B, as well as in human EGFR and *erb*B2 proteins. Furthermore, all splice junctions of the three characterized exons were conserved between *erb*B3 and *erb*B2. Amino acid sequence homology to other known tyrosine kinases was significantly lower, ranging from 39% to 46%. A single 6.2-kb-specific transcript was identified by Northern (RNA) blot analysis of human epithelial cells using the 150-bp *Spe*I-*Acc*I exon-containing fragment as probe. Under the stringent hybridization conditions used, this probe detected neither the 5-kb *erb*B2 mRNA nor the 6- and 10-kb EGFR mRNAs (data not shown). All of these findings suggested the identification of a third member of the *erb*B protooncogene family, which we have designated as *erb*B3 [15]. Thus, three distinct genetic loci in the human genome encode closely related members of the *erb*B/EGFR family, including the EGFR

gene (*erb*B) on chromosome 7p12-13 [20], *erb*B2 on chromosome 17p12-21.3[16,18], and *erb*B3 on chromosome 12q11-13 [15].

## 3. The *erb*B/EGFR subfamily of tyrosine kinases: Structural conservation and expression pattern

The complete coding structure of *erb*B2 [18,21,22] and *erb*B3 [15,19] were determined by sequence analysis of cDNA clones comprising the entire coding sequences, demonstrating the close overall structural homology of both *erb*B2 and *erb*B3 to the EGFR protein [11,23]. The colinear similarity ranging from the aminoterminus of the proteins throughout the carboxyl-terminal boundary of the tyrosine kinase domain, combined with the structural conservation of functionally essential domains between the EGF receptor, *erb*B2, and *erb*B3, defines the subfamily of human *erb*B/EGF receptor tyrosine kinases and readily distinguishes it from other tyrosine kinase subfamilies (Figure 1).

The deduced amino-acid sequences of *erb*B2 and *erb*B3 predicted single polypeptide-chain structure of membrane-anchored receptor tyrosine kinases most closely related to the EGFR (Figure 1). Conserved structural features among the three *erb*B-related receptor tyrosine kinase genes include a hydrophobic signal sequence and the transmembrane domain, which border an extracellular ligand-binding domain with amino-acid

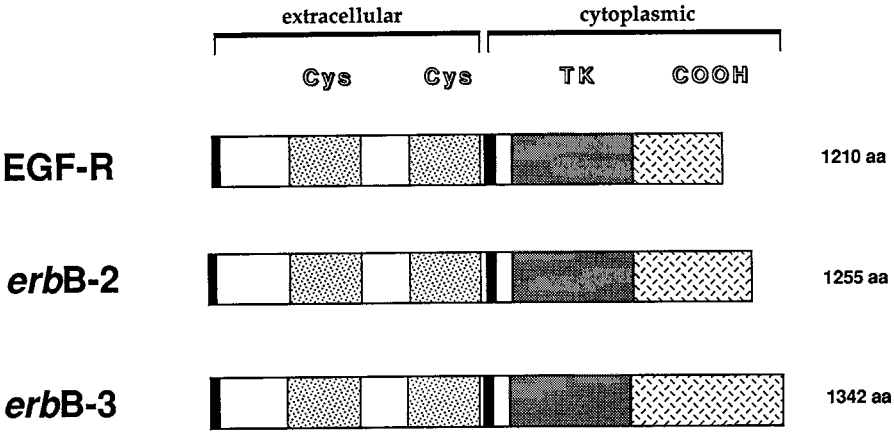



*Figure 1.* The human *erb*B/EGFR tyrosine kinase subfamily: Comparison of the predicted polypeptide structure of EGFR, *erb*B2, and *erb*B3. Hydrophobic signal sequence and transmembrane domain are depicted as solid boxes at the aminoterminus and at the junction of the extracellular and cytoplasmic coding sequences, respectively. Other structural subdomains include signature cysteine clusters (Cys) within the extracellular ligand-binding domain, the tyrosine kinase domain (TK), and the carboxyl-terminal domain (COOH) in the cytoplasmic portion of the polypeptides. Sequences used for comparative computer analysis appear in references [11,21,15].

identities in excess of 40% (for review, see [24] and references therein). The putative *erb*B3 ligand-binding domain, with 45% identical amino acid residues, is marginally more closely related to the EGFR than that of *erb*B2. Within this domain, all 50 cysteine residues of the three processed polypeptides are conserved and similarly spaced for the EGFR, *erb*B2, and *erb*B3. Forty-seven cysteine residues are organized in two clusters containing 22 and 25 cysteines, respectively, a structural hallmark of this tyrosine kinase receptor subfamily. The cytoplasmic domain harbors a contiguous tyrosine kinase domain of 277 amino acids representing the most conserved region between EGFR, *erb*B2, and *erb*B3. In this region, *erb*B3 shares 60% or 62% of amino acid identity and 90% or 89% conservation with the EGFR and *erb*B2, respectively, while EGFR and *erb*B2 exhibit an even closer homology of 80% amino acid identity. The most divergent region among EGFR, *erb*B2, and *erb*B3 is their carboxyl terminus. In fact, differences in molecular weights of their predicted precursor polypeptides of 135, 136, and 148 kDa, respectively, are accounted for by different lengths of their carboxyl-terminal coding sequence (Figure 1). Consistent with the EGF receptor and *erb*B2 proteins, where major autophosphorylation sites have been mapped to this portion of the protein, was the prediction of such sites in the carboxyl-terminal portion of the *erb*B3 coding sequence. Likewise, an ATP binding site characteristic of protein tyrosine kinases is conserved in the amino-terminal portion of the predicted tyrosine kinase domain [15].

The structural relatedness of the *erb*B2 and *erb*B3 extracellular domains with that of the EGFR raises the question of functional interaction of one or more of an increasing number of EGF-like ligands with the *erb*B2 and *erb*B3 products. A family of related growth factors, including epidermal growth factor (EGF) [25], transforming growth factor-α (TGF-α) [26], vaccinia virus growth factor [27], and amphiregulin [28], binds to the EGFR extracellular domain and triggers its intrinsic tyrosine kinase activity. The homology of these ligands is reflected in the conservation of a characteristic cysteine motif. While *erb*B2 specific ligands await molecular characterization of their primary coding structure, evidence for such molecules has been reported [29,30], including a recent description of a bifunctional *erb*B2 ligand that also interacts with the EGF receptor and shares an immunological relationship with TGF-α [30].

The normal expression pattern in human tissues [18,31] of both the EGFR and *erb*B2 genes indicated a wide range of physiological target-cell specificity for ligands, including epithelial tissues and brain. Among postnatal tissues analyzed, the *erb*B3 transcript was expressed in placenta, skin, stomach, lung, kidney, and brain, and was undetectable in fibroblasts, skeletal muscle, or lymphoid cells, thus implying preferential expression in epithelial tissues and brain. Comparative analysis of the three *erb*B receptor genes in individual cell types revealed that epithelial cells, including keratinocytes and glandular epithelial cells, coexpressed all three genes. In

| | EGFR | *erb*B-2 | *erb*B-3 |
|---|---|---|---|
| | **NORMAL EXPRESSION PATTERN** | | |
| KERATINOCYTE | + | + | + |
| GLANDULAR EPITHELIUM | + | + | + |
| FIBROBLAST | + | + | - |
| MELANOCYTE | - | + | + |
| HEMATOPOETIC | - | - | - |
| | **ABNORMALITIES IN HUMAN NEOPLASIA** | | |
| **OVEREXPRESSION (+/- GENE AMPLIFICATION)** | squamous cell carcinoma adenocarcinoma glioblastoma | adenocarcinoma (breast, stomach, ovary, lung, colon, pancreas, kidney, salivary gland) | adenocarcinoma (breast) |
| **STRUCTURAL ALTERATIONS** | glioblastoma squamous cell carcinoma | intestinal adenocarcinoma | |

*Figure 2.* Normal expression pattern and abnormalities in neoplasia of the human *erb*B/EGFR family. The normal expression pattern derives from comparative transcript analysis of EGFR, *erb*B2, and *erb*B3 genes in primary or immortalized cultures representative for the indicated cell types. (+) detectable, (−) undetectable [15]. Abnormalities in human neoplasia are compiled from the literature (for references refer to the text). Both categories of tumors with overexpression in the presence or absence of gene amplification, respectively, are listed combined. Structural alterations include abnormalities suggestive of gene rearrangement.

contrast, melanocytes and stromal fibroblasts specifically lacked EGFR and *erb*B3 transcripts, respectively [15] (Figure 2). There is evidence for autocrine [32] as well as paracrine effectors [33] of normal cell proliferation. The inherent transforming potential of autocrine growth factors, however, suggests that growth factors act on their target cell population by a paracrine route. Thus melanocytes and stromal fibroblasts may be sources of paracrine growth factors for EGFR and *erb*B3 products, respectively, that are expressed by the other cell types residing in close proximity in epidermal tissues [15].

## 4. Overexpression of *erb*B/EGFR family members in human neoplasia

The EGF receptor gene has been frequently found amplified and/or overexpressed in human malignancies, including gliobastomas and epithelial

neoplasia of both squamous and glandular types [34–38]. In some of these tumors, EGF receptor gene amplification was accompanied by gene rearrangement [11,34] (Figure 2). Furthermore, coexpression of TGF-α was observed in tumor cells exhibiting increased EGF receptor expression, providing evidence for the presence of an autocrine loop in such tumors [39,40].

The initial identification of *erb*B2 gene amplification in tissue from a primary mammary adenocarcinoma suggested the possibility that *erb*B2 overexpression might contribute to neoplastic growth in this tumor type [14]. To assess the role of *erb*B2 in human mammary neoplasia, we compared mRNAs of 16 mammary tumor cell lines to normal human fibroblasts, M413, and a human mammary epithelial derived cell line, HBL100. Increased expression of an apparently normal-size 5-kb transcript was detected in 8 of 16 tumor cell lines when total cellular RNA was subjected to Northern blot and semiquantitative dot blot analysis [41] (Figure 2).

To investigate alterations of the *erb*B2 gene associated with its overexpression, we examined the gene locus by Southern blot analysis in these same cell lines. The normal restriction pattern was detected in all DNA samples tested, indicating that gross rearrangements in the proximity of the *erb*B2 coding region had not occurred. When compared with normal human DNA, the *erb*B2-specific restriction fragments appeared amplified in several cell lines, including SK-BR-3, BT474, and MDA-MB361. Quantitation of *erb*B2 gene copy number by DNA dot blot analysis revealed a four- to eightfold *erb*B2 gene amplification in SK-BR-3 and BT474 relative to diploid human DNA and a two- to fourfold *erb*B2 gene amplification in the MDA-MB453 and MDA-MB361 cell lines. Thus, gene amplification was associated with overexpression ranging from 64- to 128-fold in the four cell lines with the highest levels of *erb*B2 mRNA. In contrast, no gene amplifcation was detected in four tumor cell lines with intermediate *erb*B2 overexpression in the range of four- to eightfold [41] (Figure 2).

In chemically induced rat neuroblastomas, a point mutation within the transmembrane region activates *neu*, the rat homologue of *erb*B2, to acquire transforming activity in the NIH/3T3 transfection assay [9]. We have reported a lack of transforming activity of a large group of human mammary tumors and tumor cell lines in this assay [42]. These included those that exhibited *erb*B2 gene amplification and/or overexpression in the absence of aberrant transcript sizes. Thus, our studies suggested that a structurally normal *erb*B2 coding sequence was overexpressed in these mammary tumor cell lines [41]. More recently, direct analysis of *erb*B2 transmembrane domains from a large group of human mammary tumors did not reveal activating mutations [43], underscoring the hypothesis that overexpression of structurally normal *erb*B2 may be frequently involved in the human neoplastic process (Figure 2). Indeed, an incidence of 10–40% *erb*B2 gene amplification in primary human breast and ovarian cancer has been observed [44–47,38]. Furthermore, there is evidence that increased *erb*B2

expression might be indicative of a more aggressive disease course in the patient [44,46,47]. In addition, *erb*B2 amplification and/or overexpression occurs at a variable incidence in adenocarcinomas of other organs, including tumors of the gastrointestinal tract, lung, kidney, pancreas, and salivary gland [17,48–50]. To date, structural abnormalities, including gene rearrangement, have been only reported at low frequency, as in the case of gastrointestinal adenocarcinomas [21,51,52] (Figure 2).

A survey of *erb*B3 expression in tumor cells detected the *erb*B3 transcript in 36 out of 38 carcinomas and in 2 of 12 sarcomas, whereas seven tumor cell lines of hematopoetic origin lacked measurable *erb*B3 mRNA. Markedly elevated levels of a normal-size transcript were observed in 6 out of 17 tumor cell lines derived from human mammary carcinomas when compared with nonmalignant control cells derived from human mammary epithelium. Southern blot analysis of these samples did not reveal alterations suggestive of gene rearrangement or gene amplification. Thus, overexpression of *erb*B3 in the absence of gene amplification in a significant subset of human mammary tumor cell lines suggests that *erb*B3 may be subjected to similar alteration mechanisms as the related EGFR and *erb*B2 genes (Figure 2). Furthermore, these findings raise the possibility that this new member of the *erb*B/EGF receptor family may also play an important role in some human malignancies [15].

## 5. Transforming potential of normal and activated *erb*B2 and EGFR in model systems

Analysis of human neoplasia suggested that quantitative alterations in the expression levels of *erb*B receptor family members might suffice to overcome normal growth regulation and in this way contribute to malignant transformation. Thus, we sought to directly assess the effect of overexpression of *erb*B2 and EGFR in NIH/3T3 cells, a system widely used for transformation studies. To this end, we engineered eukaryotic expression vectors for *erb*B2, based on the transcriptional initiation sequences of either the Moloney murine leukemia virus long terminal repeat (Mo-MLV LTR) or the SV40 early promoter in an attempt to express the *erb*B2 cDNA at different levels in NIH/3T3 cells [53]. Similarly, an LTR-based expression vector was engineered for EGFR (LTR-EGFR) [54] (Figure 3).

The LTR-based *erb*B2 expression vector (LTR-*erb*B2) induced transformed foci at high efficiencies of $2.0 \times 10^4$ focus-forming units per picomole of DNA (ffu/pM). In striking contrast, the SV40/*erb*B2 construct failed to induce any detectable morphologic alteration of NIH/3T3 cells transfected under identical assay conditions. Immunological analysis revealed that SV40/*erb*B2 transfectants expressed $gp185^{erbB2}$ at tenfold higher levels than control NIH/3T3 cells. A further tenfold increase in $gp185^{erbB2}$ expression was detected in LTR/*erb*B2 transfectants [53] (Figure 3). These

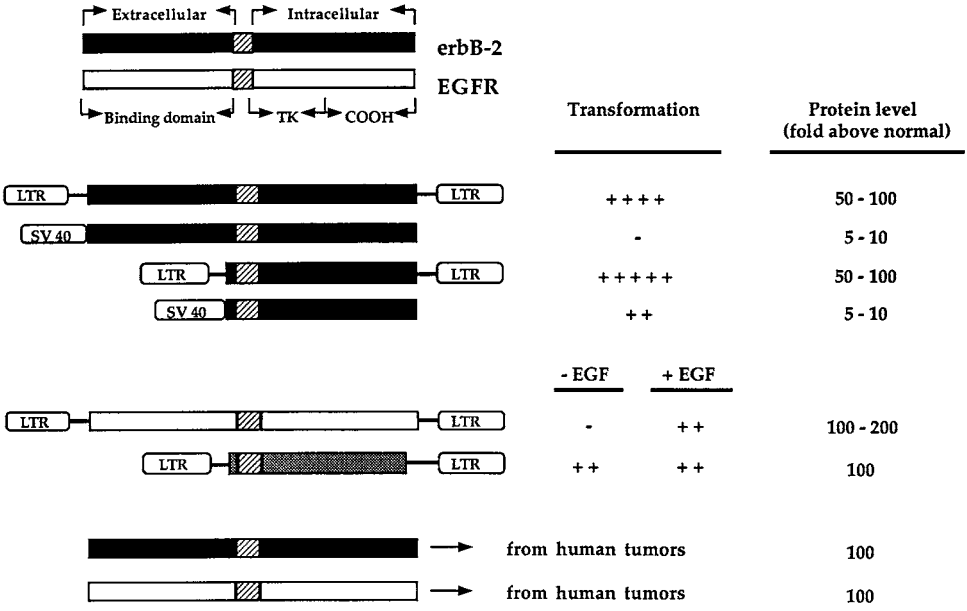



*Figure 3.* Transforming potential of *erb*B2 and EGFR overexpressed in NIH 3T3 cells. The structure of the eukaryotic expression vectors for the *erb*B2 and EGFR cDNAs used in the gene transfer experiments is depicted. Details about the engineering of the vectors can be found in Di Fiore et al. [53,54] for *erb*B2 and EGFR. Transforming potential was measured by transfection in NIH 3T3 cells. Protein levels were assayed by immunoblot with specific antibodies directed against $gp185^{erbB2}$ or EGFR, as described in Di Fiore et al. [53,54]. At the top of the figure the structures of *erb*B2 and EGFR are shown with their functional domains. At the bottom of the figure the level of overexpression of $gp185^{erbB2}$ and EGFR in human tumors is indicated, in comparison with the levels achieved in the transfectants. Filled, open, and dotted bars indicate the *erb*B2, EGFR, and v-*erb*B sequences, respectively. The transmembrane regions are always represented by a dashed box.

results demonstrated that the higher levels of *erb*B2 expression under LTR influence correlated with its ability to exert transforming activity and established a quantitative threshold for the oncogenic action of $gp185^{erbB2}$ (Figure 3). Independent research has confirmed the transforming potential of the overexpressed normal human $gp185^{erbB2}$ [55]. The LTR-EGFR expression vector was also capable of inducing malignant transformation of NIH/3T3 [54] (Figure 3). The appearance of the transformed phenotype was, however, conditionally dependent on the administration of a superphysiological concentration of EGF (around 20 ng/ml) [54,56,57]. Noteworthy, the LTR-EGFR was 100-fold less potent than the LTR-*erb*B2 expression vector ($\sim 10^2$ ffu/pM for LTR-EGFR vs. $\sim 10^4$ ffu/pM for the LTR-*erb*B2), despite comparable levels of protein expression (around $1.0 \div 2.0 \times 10^6$ receptors/cell) [54] (Figure 3). The LTR-EGFR vector, however, displayed a transforming efficiency indistinguishable from that of v-*erb*B, its avian

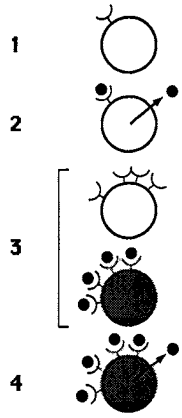
truncated-activated counterpart, engineered in an identical expression vector [54]. These findings suggested that the mitogenic signaling pathways mediating the action of *erb*B2 and EGFR must differ, at least in part, and that in NIH3T3 cells *erb*B2 couples with a more potent growth signaling pathway than the EGFR.

In order to assess the relevance of $gp185^{erbB2}$ and EGFR levels inducing in vitro transformation for *erb*B2 or EGFR overexpression in human tumors, we sought to compare the level of overexpression of these proteins in human tumor cell lines possessing amplified *erb*B2 or EGFR genes with that of NIH/3T3 cells transformed by either of the two coding sequences. By immunoblot analysis we demonstrated that human mammary cell lines that overexpressed the *erb*B2 gene displayed levels of the *erb*B2 gene product capable of inducing malignant transformation in a model system [53] (Figure 3). Similarly, levels of EGFR overexpression that conferred a strong selective advantage to NIH/3T3 cells in the presence of ligand were demonstrated in representative human tumor cell lines that contained amplified copies of an apparently normal EGF receptor gene [54] (Figure 3).

A number of other genetic alterations have been shown to be able to confer transforming potential in vitro upon *erb*B2 and EGFR. Among these, of particular interest is amino-terminal truncation. In the case of *erb*B2, an expression vector encoding a protein, Δ*Nerb*B2, devoid of the extracellular ligand-binding domain, showed increased transforming efficiency at both low and high levels of expression, as achieved under SV40 or LTR transcriptional control, respectively [53] (Figure 3). Similar results have been obtained in the rat *neu* system [58]. Interestingly, the activation of *erb*B2/*neu* by amino-terminal truncation closely resembles the activation of EGFR by a similar alteration, thus suggesting that the extracellular domains of both these proteins exert a negative regulatory influence that can be constitutively abolished by deletion or reversibly restrained in response to ligand binding.

## 6. The role of autocrine stimulation in in vivo and in vitro transformation by EGFR and *erb*B2

The described studies directly involved EGFR and *erb*B2 in the neoplastic process and established a mechanistic basis for their amplification/overexpression as representing a causal driving force in the clonal evolution of a tumor cell, rather than being an incidental consequence of tumorigenesis. Several questions, however, remained unanswered. First, overexpression of *erb*B2 itself was able to transform NIH3T3 cells without the addition of a specific growth factor. This raised the question as to the functional status of the *erb*B2 kinase in physiological conditions as well as in tumor cells. Second, although EGFR overexpression was capable of conferring a ligand-dependent growth advantage to NIH3T3 cells, the optimal EGF concentration required to obtain this effect was 20 ng/ml. These levels are well



| | Phenotype (soft agar and tumorigenicity) | Proliferation (saturation density) | Morphology | Growth in the absence of EGF in serum-free medium | |
|---|---|---|---|---|---|
| | | | | Sparse cells | Dense cells |
| 1 | Normal | Normal | Normal | - | - |
| 2 | Normal | High | Transformed | - | + |
| 3 | Normal | Normal | Normal | - | - |
| | Transformed | High | Transformed | | |
| 4 | Transformed | High | Transformed | + | + |

*Figure 4.* TGF-α-EGFR interactions in the NIH 3T3 model system. Expression vectors for EGFR [54] and TGF-α [60] were transfected into NIH 3T3 cells alone or in combination. The figure depicts the possible combinations: (1) normal NIH 3T3 cells expressing around $10^4$ EGFRs/cell and not producing TGF-α (2) NIH 3T3 cells transfected with and LTR-TGF-α vector; (3) NIH 3T3 cells transfected with an LTR-EGFR vector and expressing around $10^6$ EGFRs/cell (shown in the absence or presence of exogenous EGF stimulation); NIH 3T3 cells expressing $10^6$ EGFRs/cell and transfected with an LTR-TGF-α vector. Indications as to methodologies used for soft agar, tumorigenicity testing, and for culture in serum-free medium can be found in Marco et al. [40].

above the physiological concentration of EGF or TGF-α in the plasma of humans or rodents [25]. Moreover, NIH-EGFR cells were not tumorigenic when injected into athymic nude mice, demonstrating that ligand availability in vivo must not be sufficient to unmask a proliferative advantage conferred by EGFR overexpression [54,40]. A conceptual framework trying to resolve these questions was provided by the autocrine hypothesis of cellular transformation [59,32]. According to this hypothesis the inappropriate expression of a growth factor in a responsive cell (or vice versa) is sufficient to trigger transformation. Consequently, we investigated the role of autocrine loops in malignant transformation by EGFR and *erb*B2 in vitro and in vivo.

## 7. Mechanisms by which EGF receptor and TGF-α contribute to malignant transformation

Since a role has been postulated for TGF-α in malignant transformation, we sought to investigate the combined effect of TGF-α expression and EGFR overexpression in model systems. To this end a retrovirus engineered to encode the TGF-α molecule [60] was introduced in NIH/3T3 and NIH/

EGFR cells, and mass populations designated NIH-TGF-α and NIH-EGFR-TGF-α were established [40] (Figure 4). TGF-α expression in NIH-TGF-α cells, which express normally low levels of EGFR (around $10^4$ receptors/cell), was not able to induce transformation as assessed by the ability to grow in anchorage-independent conditions and tumorigenicity in athymic nude mice. However, NIH-EGFR-TGF-α cells (expressing $1.0–2.0 \times 10^6$ EGFRs/cell) displayed a high clonogenic capacity and a short latency for tumorigenicity in mice (Figure 4). We concluded, therefore, that the availability of a critical number of EGFR was needed for TGF-α to exert its transforming action in this model system. Interestingly, NIH-TGF-α cells, despite their lack of transformation (as assessed by clonogenic ability and tumorigenicity tests) displayed altered morphology in vitro and increased saturation density (Figure 4). We postulated that the proliferative advantage conferred by TGF-α in this situation (at low levels of EGFR expression) was not strictly cell associated (hence the lack of clonal proliferative advantage) but rather was due to a feeding effect of the secreted TGF-α on the entire culture. Indeed, in stringent serum-free growth conditions, while normal NIH/3T3 or NIH/EGFR cells required both insulin and EGF for proliferation, NIH-TGF-α cells grew in the absence of EGF only when plated at high cell density and did not proliferate at a low cell concentration. Conversely, NIH-EGFR-TGF-α cells proliferate in the absence of EGF both at high and low seeding concentrations. Thus, while NIH/TGF-α cells were stimulated by a 'paracrine' feeding mechanism, NIH-EGFR-TGF-α cells were transformed by an autocrine mechanism involving TGF-α stimulation of the producing cell [40] (Figure 4).

On the basis of the above evidence and preexisting literature [39], it was tempting to hypothesize that coexpression of TGF-α and high levels of EGFR are concomitantly required to contribute to tumor development in vivo. If so, then the two events should be coselected in human malignancies. To test this hypothesis we analyzed the levels of expression of TGF-α mRNA on two panels of human tumor cell lines, matched for histopathological derivation, known to express high or normal levels of EGFR. A striking correlation was found between coexpression of TGF-α and high levels of EGFR in cell lines derived from glioblastomas, squamous carcinomas, and mammary adenocarcinomas [40].

At the biochemical levels, we analyzed the state of in vivo EGFR tyrosine phosphorylation, which is known to correlate well with its biological activity. Even in cells overexpressing around $2.0 \times 10^6$ EGFRs/cell, like NIH-EGFR, receptor activation was tightly controlled. NIH-EGFR cells, in fact, only displayed autophosphorylation on tyrosine residues upon EGF triggering, while no EGFR tyrosine phosphorylation was detectable in the absence of EGF. Under the latter conditions, however, NIH-EGFR-TGF-α cells showed readily detectable levels of P-Tyr-containing EGFR [40]. Based on these results, in human tumor cells overexpressing EGFR and TGF-α one should expect evidence of constitutive EGFR tyrosine phosphorylation

(i.e., in the absence of external ligand addition) if the autocrine loop resulted in chronic activation of the EGF-responsive mitogenic pathway. Indeed representative human tumor cell lines selected on the basis of their coexpression of TGF-α and high levels of EGFR displayed high constitutive levels of P-Tyr-containing EGFR [40]. These results argued that TGF-α expression by EGFR overexpressing tumor cells has functional implications for tumor cell growth and demonstrated that a quantitative requirement for both ligand and receptor expression has to occur for the institution of a transforming autocrine loop.

## 8. The normal *erb*B2 product is an atypical receptor-like tyrosine kinase with constitutive activity in the absence of ligand

The mechanisms by which high levels of *erb*B2 product cause transformation also remain to be elucidated. A characteristic feature of gp185$^{erbB2}$ overexpressed by the gene-transfer technique in different model systems (fibroblastic, hematopoietic, epithelial) or naturally in tumor cells is to possess constitutive (i.e., in the apparent absence of ligand) levels of tyrosine phosphorylation [61]. This is due to gp185$^{erbB2}$ autophosphorylation, rather than phosphorylation by another cellular kinase. In fact, a kinase-inactive version of gp185$^{erbB2}$, with a mutation in its ATP-binding site, was shown to be devoid of transforming activity in NIH/3T3 cells and also lacked tyrosine autophosphorylation in vitro [62,63]. The apparent constitutive activity of gp185$^{erbB2}$ might be explained by different models. One possibility is that under standard culture conditions a putative *erb*B2 ligand chronically stimulates gp185$^{erbB2}$. Such a ligand could be either present in the serum of culture medium or be produced in an autocrine fashion. Alternatively, the normal gp185$^{erbB2}$ might be an atypical receptor-like tyrosine kinase endowed with constitutive catalytic activity.

Two lines of evidence argued against the presence of a putative ligand for *erb*B2 in serum. First, NIH-*erb*B2 cells retained their transformed phenotype when cultivated in the absence of serum. Conversely, individual isolated transformed foci, induced in NIH/3T3 cells by EGFR overexpression and exposure to EGF, promptly reverted to a stable, flat morphology when subcultured in a chemically defined medium in which no EGF was available. Furthermore, the state of gp185$^{erbB2}$ tyrosine phosphorylation was unaffected by serum deprivation, and the half-life of pulse-labeled gp185$^{erbB2}$ was not influenced by the presence or the absence of serum in the chase medium [61]. Thus, based upon all of the above evidence, we concluded that the constitutive activity of gp185$^{erbB2}$ overexpressed in NIH3T3 cells was not due to the presence of a ligand in serum.

Direct testing of autocrine production of an *erb*B2 ligand was impossible, since such a molecule had not been identified at the time. As an alternative approach we tested for the presence of an *erb*B2 ligand with chimeric

molecules containing the extracellular domain of gp185$^{erbB2}$ and the intracellular domain of EGFR. If an *erb*B2 ligand were produced by NIH/3T3 cells, it should activate such an *erb*B2/EGFR chimera in an autocrine fashion and thus be detectable in transformation and autophosphorylation assays in vivo. These chimeric molecules, however, were found unable to transform NIH/3T3 cells and did not display any constitutive levels of tyrosine phosphorylation, while they were responsive in terms of autophosphorylation and mitogenesis to an agonist-like monoclonal antibody directed against the extracellular domain of *erb*B2, thus establishing their functional integrity. Consequently, autocrine production of a putative ligand by NIH/3T3 cells is not responsible for the constitutive activity of gp185$^{erbB2}$ overexpressed in NIH3T3 cells [61].

We finally searched for the basis of the gp185$^{erbB2}$ constitutive activity. To this end, we engineered chimeric molecules possessing the extracellular domain of EGFR and the intracellular domain of *erb*B2 (EGFR/*erb*B2 chimerae). Interestingly, when expression vectors for these chimerae were transfected in NIH/3T3 cells, they readily displayed transforming activity in the absence of EGF supplementation. This phenomenon was paralleled at the biochemical level by constitutive autophosphorylation activity on tyrosine residues. The EGFR/*erb*B2 chimerae, however, retained EGF responsiveness, and EGF treatment further increased their transforming as well as autophosphorylation activities [61]. The constitutive tyrosine phosphorylation of the EGFR/*erb*B2 chimera contrasted the undetectable levels of tyrosine phosphorylated EGFRs under the same conditions. Thus, the cytoplasmic domain of *erb*B2 must possess the critical determinants that impart to normal gp185$^{erbB2}$ its ligand-independent activity. Additional studies involving chimaerae at the COOH domain revealed that these determinants are localized within the last 300 amino acids of the COOH domain of *erb*B2 [64].

While our results establish that under conditions of high-level overexpression a ligand is not required for *erb*B2 to transform an in vitro model system, they do not preclude the existence of such a molecule. Indeed, ligand-like activities for *erb*B2 have been reported [29,30]. Furthermore, it is well established that genetic manipulations, such as certain transmembrane mutations or amino-terminal truncations, can enhance gp185$^{erbB2}$ kinase and transforming activity. Thus, even when overexpressed, *erb*B2 is not maximally activated and might be further stimulated by a putative ligand. In addition, the constitutively high levels of gp185$^{erbB2}$ activity raise the intriguing possibility that its activity might be subjected to negative ligand modulations.

## 9. Multiple levels of regulation of EGFR and *erb*B2 mitogenic signaling

A line of investigation made feasible by the availability of eukaryotic expression vectors for EGFR and *erb*B2 focused on the introduction of

foreign receptors into naive cell lines, i.e., cell lines that do not normally express EGFR or *erb*B2. We chose this approach to be able to discriminate whether the sole determinant responsible for the mitogenic response to a particular growth factor is the expression of its specific receptors or whether other intracellular determinants also participate in this regulation process. We elected, for this purpose, to use the hematopoietic cell line 32D. These cells are committed-undifferentiated myeloid precursors, they do not display tumorigenicity in nude mice, and they lack endogenous EGFR or *erb*B2. In addition, 32D cells are absolutely dependent for growth and survival on IL-3. Upon expression of EGFR in these cells, they could be cultivated in medium lacking IL-3 and containing EGF. In addition, expression of v-*erb*B, the activated counterpart of EGFR, abrogated 32D cells from their IL-3 dependency, consistent with the constitutively activated state of the v-*erb*B kinase [65]. Thus, an EGF-responsive mitogenic signaling pathway existed in 32D cells that could be recruited upon EGFR expression. This study identified a major mechanism of control of mitogenic signaling at the level of receptor expression on the cell surface [65].

It is not clear whether the EGF-responsive pathway present in 32D cells is physiological for EGFR. One could argue that an ectopically expressed

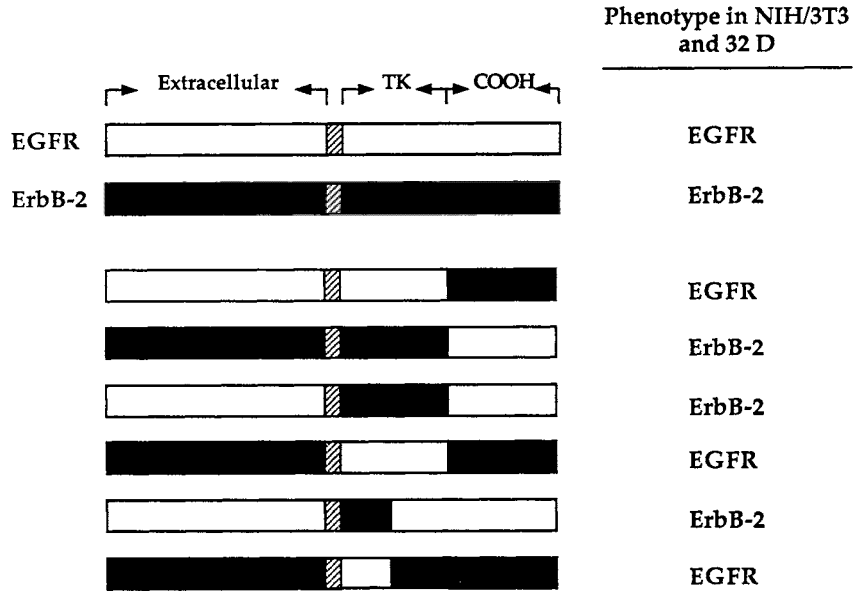



*Figure 5.* Identification of the domain responsible for the differential coupling of *erb*B2 and EGFR with the mitogenic signaling pathway in NIH 3T3 and 32D cells. Chimeric molecules between different domains of *erb*B2 and EGFR are depicted in comparison to their parental molecules. Details about the engineering of the relevant eukaryotic expression vectors can be found in Di Fiore et al. [64,66]. The EGFR phenotype was defined on the basis of efficient mitogenic coupling in 32D as compared to relatively weak coupling in NIH 3T3. Conversely, the *erb*B2 phenotype indicates potent coupling in NIH 3T3 and weak activity in 32D cells. Filled and open bars indicate *erb*B2 and EGFR sequences, respectively.

receptor might be responsible for abnormal coupling with intermediates of other mitogenic signaling pathways. However, when the dose–response curve to EGF was analyzed in 32D-EGFR cells in comparison to physiologically EGF-responsive cells (NIH-EGFR), no difference could be detected in the EGF $ED_{50}$ for mitogenesis (~50–100 pM in both cases). These results constitute strong evidence in favor of coupling of the foreign receptor with a genuine intracellular signaling pathway. Otherwise, one would expect a lower affinity of interaction between EGFR and nonphysiological substrates, which should be reflected in a higher EGF $ED_{50}$ for mitogenesis.

When gp185$^{erbB2}$ was expressed in 32DS cells it was found unable to couple with mitogenic signaling pathways [66], in spite of the expression levels comparable to the EGFR levels in 32D-EGFR. In addition, gp185$^{erbB2}$ expressed in 32D-*erb*B2 cells displayed constitutive autokinase activity comparable to that in NIH-*erb*B2 cells, where it acts as a potent transforming gene and mitogenic signal transducer [53,66]. Even activated forms of the *erb*B2 product, such as Δ*erb*B2 (see before and [53]) or *erb*B2Glu$^{659}$ [67], triggered only weakly mitogenic signals in 32D cells, despite maximal activity in fibroblast target cells [53,66]. These findings indicated that the regulation of gp185$^{erbB2}$ mitogenic potency is, at least in part, exerted at the level of intracellular signal transduction through the availability of critical substrates. Our findings further implied that this mechanism might be relevant in controlling the activity of the constitutively active *erb*B2 kinase under physiological conditions.

## 10. EGFR and *erb*B2 couple with distinct mitogenic pathways: Identification of the structural determinant responsible for differential coupling

The aforementioned results also established that EGFR and *erb*B2 must couple with, at least in part, distinct mitogenic signaling pathways. gp185$^{erbB2}$ was in fact 100-fold more potent than EGFR, expressed at comparable levels, at inducing transformation of NIH/3T3 cells [66,54]. Conversely, gp185$^{erbB2}$ exhibited an insignificant ability to stimulate 32D cells, whereas EGFR was able to efficiently couple with mitogenic transduction pathways in these latter cells [65,66]. The combined usage of these two cell systems provided a tool to identify determinants in the primary structure of EGFR and gp185$^{erbB2}$ responsible for the coupling with different mitogenic pathways [66,68] (Figure 5). We approached the problem by engineering a series of chimeric molecules between domains of the intracellular portions of EGFR and *erb*B2, and expressed these molecules in NIH/3T3 and 32D cells (Figure 5). Surprisingly, the reciprocal switching of the COOH domains, which display the highest level of dissimilarity, was not able to shunt the catalytic activity of either receptor to an alternate cytoplasmic signaling pathway. Instead, the specificity for signal transduction was shown to reside in the highly conserved TK region, and in particular in its juxtamembrane

position [66,68]. Thus, this subdomain may represent a subfamily-specific 'variable' region responsible for specific substrate coupling.

## 11. Implications

In human neoplasia, both *erb*B/EGFR and *erb*B2 have been frequently implicated in the neoplastic process, predominantly by overexpression of an apparently normal coding sequence. At the high levels of overexpression observed in the presence of gene amplification, mechanistic in vitro studies have demonstrated that both *erb*B2 and EGFR are capable of conferring the malignant phenotype onto NIH3T3 cells. While in the case of the EGFR this is conditionally dependent on stimulation of a ligand, structurally normal *erb*B2 exerts its oncogenic potential in the absence of a ligand under these conditions, due to an abnormally high intrinsic tyrosine kinase activity. Studies of human mammary tumor cell lines suggested early on that two groups of tumors with *erb*B2 overexpression can be identified comprising moderate levels and high levels of overexpression, respectively. While the latter was unequivocally associated with gene amplification, the former did not reveal genetic abnormalities indicative of gene amplification or gene rearrangement. More recently, accumulating evidence confirms the frequent occurrence of moderate *erb*B2 overexpression in the absence of gene amplification in primary breast carcinomas [46,47,69,38]. In model systems, levels of $gp185^{erbB2}$ achieved with SV40 promoters and comparable to those detected in the moderate expressor group produced the oncogenic phenotype only when paired with structural alterations of the coding sequence. Based upon the apparent lack of structural *erb*B2 alterations in human mammary tumors, it is tempting to hypothesize that in naturally occurring tumors autocrine and/or paracrine *erb*B2-ligand stimulation might be required for $gp185^{erbB2}$ to confer a proliferative advantage when expressed at intermediate levels. The discoveries of *erb*B2 ligand activities render investigations of this concept an attractive task. Furthermore, elucidation of distinct mechanisms for *erb*B2 activation in human tumor cells would be fundamental for attempts to revert the neoplastic phenotype by virtue of interference with distinct activations of the *erb*B2-mediated transforming signaling pathway. Finally, the observation that a third member of the *erb*B/EGFR subfamily, *erb*B3, exhibits increased transcript levels in a subset of human mammary tumor cell lines in the absence of detectable gene amplification or rearrangement suggests that this more recently identified member of an expanding growth-factor receptor subfamily may be involved in certain human epithelial malignancies.

## Acknowledgments

We wish to thank Stuart A. Aaronson for continuous support and encouragement. We also want to acknowledge the determinant contributions of C. Richter King, Jacalyn H. Pierce, and Oreste Segatto to the work reviewed in this chapter.

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# 8. c-*erb*B2 amplification and overexpression in human tumors

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## 1. The c-*erb*B2 protooncogene and protein

c-*erb*B2 or HER2/*neu* was originally isolated because of its close homology to the epidermal growth factor receptor (EGFr) [1,2] and, together with the recently characterized c-*erb*B3, these molecules form the type 1 family of tyrosine kinase growth factor receptors [3,4]. The epidermal growth factor receptor and possibly the other two molecules are activated by binding of ligand to their extracellular domain, which is thought to induce a conformational change allowing dimerization of two molecules. This in turn activates the tyrosine kinase of the cytoplasmic region, which phosphorylates both the receptor itself and a collection of intracellular secondary messenger substrates, thus transducing the mitogenic signal [5]. There are a number of molecules that have been shown to have agonism for the EGF receptor; however, only one putative ligand has been isolated for c-*erb*B2 — gp30 [6]. This 30-kDa protein will stimulate autophosphorylation of $p185^{erbB}$, the 185-kDa protein encoded by the c-*erb*B2 gene. Paradoxically this is not associated with cell growth when gp30 is added to cultured cells bearing a low to normal number of receptors. Addition of gp30 to cells with overexpression results in inhibition of growth, as is seen in EGF receptor overexpressing cells treated with EGF. In separate experiments conditioned media from *ras* transformed fibroblasts could induce both $p185^{neu}$ phosphorylation and a proliferative response in hemopoietic cells previously transfected with the rat c-*erb*B2 homologue called *neu*. The active component of the conditioned media has not been isolated [7].

$p185^{erbB}$ is found to be widely expressed in cells derived from all three germ layers of the human fetus as well as the placenta [8]. In adult tissues expression is localized principally to the membranes of epithelia, such as the gastrointestinal, respiratory, reproductive, and urinary tracts, and breast epithelia [9]. The sensitivity of detection for p185 by immunocytochemistry varies with the method of tissue fixation and the antibody used; thus some differences in the reported distribution may be found in the literature [8–10].

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

## 2. Mechanisms of activation of c-*erb*B2 to an oncogene

The classical mechanisms of protooncogene activation include chromosomal translocation, mutation, and amplification. Translocation, such as with the Philadelphia chromosome found in 90% of chronic myeloid leukaemias [11], has not been described for c-*erb*B2. However, mutation of the rat equivalent molecule, called *neu*, gave the first indication of its having transforming activity. The offspring of rats treated with ethylnitroso urea during pregnancy were found to develop neuroblastomas and glioblastomas [12]. DNA isolated from such tumors was found to contain an oncogenic *neu* gene that differed from the protooncogene by a point mutation in the predicted single transmembrane region. The mutation causes a substitution of glutamic acid for valine at position 664 [13]. The mutation is site specific and is limited to negatively charged residues glutamic acid and aspartic acid, which would be protonated in the membrane, and glutamine. Substitution for a basic amino acid, histidine and lysine, or a neutral, small residue, glycine, is without effect [14].

The protein encoded by the transforming gene has a greater propensity to dimerize [15] and has constitutively active tyrosine kinase [16], which has been shown to correlate with its transforming ability [17]. A model of how this simple amino acid change could stabilize the receptor's dimeric form has been proposed [18]. Experimental mutation of the human c-*erb*B2 gene to replace the equivalent valine at position 659 with glutamic acid also produces a transforming gene, both for mouse fibroblasts [19] and transgenic mice [20]. However, as yet no such point mutation has been found in any of 100 primary human breast tumour specimens examined [21] or in other tumours [22–24].

More extensive mutation with deletion of the extracellular domain, as is seen in the viral oncogene v-*erb*B, will cause activation [19,25]. However, such changes should be detected as a rearrangement on Southern blotting of human tumor DNA probed for c-*erb*B2. Such rearrangements are extremely rare, with occasional reports in colonic cancer [26] and in less than 2% of breast cancers examined [27]. It seems, therefore, that if c-*erb*B2 plays a role in human malignancy it is not by translocation or mutation but by the mechanism of amplification. Indeed initial studies of human adenocarcinomas by Southern blot revealed a proportion with amplified levels of c-*erb*B2 [27–29]. Subsequently this has been confirmed in larger series of cases where over 20% of human mammary tumors displayed additional gene copies. Amplification is always associated with an aberrantly high expression of $p185^{erbB}$ detected either as mRNA by Northern blot or as intense tumor cell membrane staining by immunocytochemistry [30]. There are, however, some primary tumor biopsies and tumor-derived cell lines that have increased expression in the absence of amplification, indicating a loss of transcriptional control as an alternative mechanism to achieve c-*erb*B2 overexpression [31,32]. Experimentally induced overexpression of c-*erb*B2

in mouse fibroblasts is transforming [25,33], as is overexpression of non-mutated rat *neu* [34]. In transgenic mice, c-*erb*B2 will cause tumors to develop in some of the tissues in which it is expressed, e.g., lymphoid tissues. However, this model does not represent the same sites of expression as are found in human tissues, and so these tumors do not correlate with human tumors, such as breast adenocarcinoma associated with c-*erb*B2 overexpression [20].

The rest of the chapter will collate information on the amplification, assessed by Southern blot, or overexpression determined by Northern blot or immunocytochemistry, of c-*erb*B2 in various tumors. Most of the overexpression is limited to adenocarcinomas. There has been some controversy over the sensitivity of each assay. Assessment of amplification by Southern blot may underestimate the number of tumors with overexpression, firstly, because it will miss those with overexpression without amplification and, secondly, through dilution of tumor tissue by adjacent stroma. In addition, care must be taken that good-quality, high molecular weight DNA is analyzed, since DNA degradation may lead to false-negative assessments of amplification [35]. Northern blot and Western blot analyses also suffer the problems of dilution of normal tissue and sample degradation. Immunocytochemistry has the advantage of localizing overexpression to the tumor cells, but suffers with variability between antibodies and sensitivity following different fixation techniques [32,36]. Also there has been some debate as to the importance of cytoplasmic staining, which is thought by some to represent antibody reactivity with a 155-kDa mitochondrial protein of unknown function or relationship to p185 [37]. Thus membrane staining as associated with amplification is now generally accepted as a positive indication for c-*erb*B2 overexpression.

## 3. Prevalence of overexpression in different human tumor types

### *3.1. Breast cancer*

c-*erb*B2 was first found to be amplified in a human mammary tumor [1]. Since then there have been an extensive number of studies on archival or fresh tumor biopsies. The overall prevalence of c-*erb*B2 amplification or the associated overexpression of membrane staining is approximately 20%. For the references listed in this chapter, the figures are 1967 overexpressing cases out of 9189 examined, giving a percentage of positive cases of 21.4% [27,30,32,38–89]. In benign tumors overexpression is very rare (3 of 149 cases in one study [42]). However, in malignant but noninvasive disease of some histological subtypes, the prevalence is very much higher. In ductal carcinoma in situ of comedo type, over 90% of tumors are found to overexpress c-*erb*B2, whereas other subtypes, such as cribriform, solid, or papillary, very rarely do [46]. Similarly, essentially all cases of mammary

Paget's disease express the protein [90]. The proportion is much less in extramammary Paget's disease, with one group reporting 40% positivity in a group of ten cases examined [91] and another no positive cases in a group of nine [92], thus supporting the hypothesis that mammary and extramammary Paget's disease have different histogenesis. The expression of c-*erb*B2 in breast adenocarcinoma is limited to ductal carcinoma and lobular carcinoma is very rarely positive [93,94].

Characteristics of c-*erb*B2 overexpressing breast tumors include an inverse correlation with estrogen receptor and progesterone receptor content [49–51,53,55,58,68,70,73,75,84,86], a higher incidence in inflammatory tumors [50,54], higher mitotic activity as assessed by Ki67 staining [36,71], and a positive correlation with tumor grade [32,43–45,58,59,63, 71,72,77,88], tumor size [27,46,70,77,89], and young age [27,64,70]. There is some variability in the reported strength of association of these factors with c-*erb*B2 overexpression, but the order in which they are listed represents a summary of the published order of significance. All the factors listed are indicative of aggressive disease, and their association with c-*erb*B2 overexpression could indicate a prognostic role for $p185^{erbB}$ expression. Some direct evidence for this comes from experiments in which overexpressing human breast cancers have been transplanted into nude mice where the aggressiveness of disease behavior correlated with c-*erb*B2 levels [95]. The most strongly predictive factor of reduced disease-free interval and overall survival is nodal status at the time of initial surgery. c-*erb*B2 positivity is also associated with nodal involvement [27,43,44,49,50,68,70,77,78,80,83,86]. Table 1 lists a collection of publications that have reported on the presence or absence of a significant prognostic role for c-*erb*B2.

As can be seen from Table 1, Slamon [27] and Varley [40] initially proposed a possible predictive power for c-*erb*B2 amplification in indicating reduced overall survival from breast cancer. The larger study by Slamon found c-*erb*B2 amplification of greater than five gene copies to be independently and strongly predictive of both death and recurrence in node-positive patients [27]. Since that report in 1987, the controversy as to the strength of $p185^{erbB}$ expression as a prognostic indicator has been debated. However, with increasingly large studies being undertaken, there seems to be little doubt that c-*erb*B2 overexpression in node-positive patients is predictive of a poor outcome independently of other prognostic indicators [30,49,59,70, 80,88,89]. Other reports have found c-*erb*B2 overexpression to be predictive in certain subgroups, such as in large primary tumors [61], estrogen receptor negative tumors [68] within the good nuclear grade subgroup [72], and with coamplification with c-*erb*A [85]. Doubt remained, however, about the prognostic significance of c-*erb*B2 in node-negative patients. As nodal status remains the strongest predictor of reduced survival, by definition node-negative patients have fewer relapses. However, two recently published retrospective studies have collated a large number of patients and found that c-*erb*B2 overexpression confers a worse overall survival [80] or is

associated with a more rapid onset of recurrence [82], independently of nodal status. Dykins et al. also showed a survival difference for c-*erb*B2-positive, node-negative, but not node-positive, patients after 12 years follow-up [87]. However, in two recent studies the authors report a prognostic role for c-*erb*B2 independent of stage and thus predictive of a poorer outcome in both node-negative and node-positive patients [88,89]. Others have not found overexpression to be associated with disease outcome in node-negative patients [76]. Some groups have shown that amplification is associated with haematogenous spread [73], but levels of expression are similar in primary tumor and metastases [54,65], indicating that c-*erb*B2 may have a role early in the development of breast cancer.

This role of c-*erb*B2 overexpression in tumorigenesis offers a potential therapeutic target for the treatment of breast cancer. Monoclonal antibodies against the extracellular domain of rat *neu* can downregulate the receptor and inhibit anchorage-independent growth [96]. Monoclonal antibodies to the c-*erb*B2 protein could be used as potential inhibitors of human cancer growth. Some preliminary tumor imaging studies have shown successful targetting in the mouse zenograft model [97], and in vitro studies on human breast cancer cell lines show inhibition of growth when treated with antibody [98]. Thus the use of such monoclonal antibodies as therapeutic agents in human breast cancer are now being assessed in Phase I clinical trials.

### *3.2. Ovarian cancer*

Slamon followed up his studies on breast cancer by screening a range of human tumors for c-*erb*B2 amplification. Interestingly, he found a proportion of ovarian tumors had increased copy number with 26% amplification in primary ovarian tumors [30]. This correlated closely with protein overexpression on tumor cell membranes, although of the total exhibiting overexpression 12% had no detectable gene amplification, a figure similar to that found in breast cancer specimens. Both increased copy number and overexpression by immunohistochemistry correlated closely with reduced overall survival. Similar levels of amplification [99,100] and overexpression [101,102] have now been described in other studies. Immunohistochemical studies have shown p185 to be expressed on serous, mucinous, and endometrioid, but not in five clear-cell, tumors studied [101] and have confirmed an association between positive membrane staining and reduced disease-free interval and overall survival [102].

**3.2.1. Other gynecological cancers.** In one report [103] of 70 endometrial and cervical biopsies, which included normal, dysplastic, and invasive carcinoma specimens, 2 of 11 endometrial and 3 of 8 cervical carcinomas had increased mRNA levels by Northern blotting. However, immunohistochemical studies revealed staining of glandular tissue in a heterogeneous

*Table 1.* c-*erb*B2 as a prognostic indicator in breast cancer

| Significant | | | | | | | Nonsignificant | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Author [REF] | Prevalence (%) | | Node-positive prevalence (%) | | Node-negative prevalence (%) | | Author [REF] | Prevalence (%) | | Node-positive prevalence (%) | | Node-negative prevalence (%) | |
| Slamon [27] | 53/189 (28) | — | 44/133 (33) | OS $p < 0.02$ DFS $p < 0.001$ | 4/34 (12) | — | Ali [38] | 12/122 (10) | NS | — | — | — | — |
| | | | | | | | Gusterson [42] | 14/103 (14) | NS | 9/57 (16) | — | 5/57 (11) | — |
| | | | | | | | Barnes [45] | 17/195 (9) | NS | 5/93 (5) | — | 12/102 (12) | — |
| Varley [40] | 7/37 (19) | OS $p < 0.0002$ | 6/25 (24) | — | 1/9 (10) | — | Van de Vuver [46] | 27/189 (14) | NS | 10/80 (13) | — | 17/109 (16) | — |
| Zhou [44] | 15/86 (17) | DFS $p = 0.06$ | 8/37 (22) | — | 1/21 (5) | — | Zhou [52] | 17/157 (11) | DFS NS | 10/89 (11) | — | 6/68 (9) | — |
| Tandon [49] | 118/728 (16) | — | 59/350 (17) | OS $p < 0.001$ DFS $p < 0.014$ | 58/378 (16) | NS | Ro [60] | — | — | — | — | 13/66 (20) | NS |
| | | | | | | | Thor [61] | 47/313 (15) | Trend | 16/120 (13) | — | 15/141 (11) | — |
| | | | | | | | Richner [67] | 10/79 (13) | NS | — | — | — | — |
| | | | | | | | Borg [70] | 52/310 (17) | — | 22/120 (18) | Trend | 20/189 (12) | NS |
| Wright [51] | 31/185 (17) | OS $p = 0.04$ DFS $p = 0.025$ | 13/62 (21) | — | 11/44 (25) | — | | | | | | | |
| Tsuda [56] | 28/148 (16) | OS $p = 0.001$ DFS $p = 0.001$ | 20/104 (20) | — | 8/72 (11) | — | | | | | | | |

| | | | | | | |
|---|---|---|---|---|---|---|
| King [57] | 19/57 (20) | DFS<br>p = 0.016 | 7/33 (21) | — | 3/21 (14) | — |
| Slamon [30] | 146/526 (28) | — | 101/345 (27) | OS<br>p < 0.045<br>DFS<br>p < 0.006 | 45/181 (25) | NS |
| Walker [59] | 14/85 (17) | OS<br>2.97 *RR<br>DFS<br>3.85 | 15/58 (26) | — | 5/27 (19) | — |
| Tsuda [63] | 27/176 (15) | OS<br>p = 0.02 | 19/104 (18) | — | 8/72 (11) | — |
| Paik [72] | 62/292 (21) | OS<br>p = 0.004 | 27/108 (25) | — | 34/182 (19) | — |
| Gullick [80] | 103/483 (21) | OS<br>p = 0.02<br>DFS<br>p = 0.007 | — | — | — | — |
| Paterson [82] | — | — | — | — | 27/230 (11) | DFS<br>p = 0.006 |
| Dykins [87] | 41/187 (22) | OS<br>p < 0.001 | — | NS | — | OS<br>p < 0.001 |
| Lovekin [88] | 75/497 (15) | OS<br>p = 0.0003 | — | OS<br>p = 0.003 | — | OS<br>p = 0.23 |
| Winstanley [89] | 104/465 (22) | OS<br>p = 0.06 | 41/165 (25) | NS | 62/294 (21) | OS<br>p < 0.03 |

Summary of papers published between 1981 and the beginning of 1991 on the use of c-*erb*B2 as a possible prognostic indicator in human breast carcinoma. For each report is listed the incidence of amplification, the percentage in parenthesis, and the power of its association with overall survival (OS) or disease-free survival (DFS) after correction for other prognostic indicators listed. Where the data are available for the node-positive and node-negative subgroups, they are included.

distribution in the majority of samples, implying possible nonspecific proliferative and/or differentiation-associated events. In this study expression of c-*erb*B2 in squamous epithelia was found to be at or below baseline levels. However, in 1 of 34 squamous cervical carcinomas studied by Berchuck et al. [104], there was evidence for c-*erb*B2 overexpression by immunocytochemistry. More studies are thus warrented on these tumor types.

### *3.3. Gastrointestinal tract*

**3.3.1. Stomach cancer.** The association of c-*erb*B2 amplification with stomach cancer was noted in one of the first studies of this protooncogene in human adenocarcinomas [28]. Subsequent series report an average prevalence of approximately 11% overall for amplification or overexpression with a range of 6–21% [26,28,29,81,105–112]. As is found in breast cancer, there is a strong correlation between amplification and mRNA levels [106] and membrane staining, although the latter may occur in the absence of amplification [111,112]. Particular histological subgroups have an increased frequency of c-*erb*B2 amplification, with up to 40% of tubular adenocarcinomas having a raised gene copy number [105]. Immunohistochemical staining is also confined to the intestinal histological group and can vary in intensity [107,108,111]. Such immunohistochemical studies show that staining is confined to the epithelial cells and is often patchy or focal [107, 108,111], which, together with the observation that elevated c-*erb*B2 levels are associated with a more advanced stage of disease [113], would imply that overexpression occurs late in the progression of stomach carcinogenesis.

Recently, the role of c-*erb*B2 as a prognostic indicator for primary stomach cancer has been investigated [108,109]. Yonemura et al. [108] found that c-*erb*B2-positive staining of 260 primary gastric cancers correlated with tumor size, serosal invasion, and nodal status. Follow-up for up to 10 years showed a significantly worse prognosis for c-*erb*B2-positive advanced cancer patients, and analysis by Cox proportional hazards model proved c-*erb*B2 to be an independent prognostic indicator of poor overall survival. However, Jain et al. [109] stained 93 primary gastric cancers and found, with a similar prevalence of overexpression (11% vs. 11.9%) and a similar period of follow-up, that c-*erb*B2 positivity conferred a survival advantage. Further studies will need to be undertaken to resolve this contradiction.

**3.3.2. Colorectal cancer.** The amplification or overexpression of c-*erb*B2 in colorectal cancers is much less common than in stomach cancer, with only just under 4% of tumors studied showing amplification [26,28,114,115] and up to 20% showing overexpression [114,115]. In two studies, in 3 out of 4 cases investigated, there was gene rearrangement [26,28]. Further studies have not confirmed this finding, but the numbers studied so far are small. There have been no reports of association with prognosis [114]. The colon

provides an excellent model for studying tumor progression, as preneoplastic polyps are easily biopsied. D'Emilia et al. [115] took advantage of this to investigate any possible role for p185 in colorectal tumor development. They found amplification in 3 out of 44 tumors and overexpression by membrane staining in eight. Twenty-three of 27 preneoplastic polyps had possible c-*erb*B2 membrane staining. However, the normal colonic mucosa stains positively on its luminal surface, and normal mucosa adjacent to tumors has additional positive membrane staining, extending into the crypts, indicating a disturbance of normal c-*erb*B2 expression in early colonic neoplasia but not necessarily a primary causative role.

### 3.3.3. Associated tissue

*Salivary gland tumors.* The prevalence of c-*erb*B2 amplification or overexpression in salivary gland tumors is very infrequent [116,117]. In a relatively large series of 131 tumors of different histological types [117], only five had evidence for c-*erb*B2 overexpression, the majority being poorly differentiated adenocarcinomas. With such a small positive series and variable histology within the group, no particular characteristics could be ascribed to the c-*erb*B2-positive group.

*Pancreatic cancer.* In a series of 87 pancreatic adenocarcinoma specimens investigated immunohistochemically, 17% had cytoplasmic staining, whereas only 2% had the positive membrane staining characteristically associated with gene amplification, suggesting that significant c-*erb*B2 overexpression is rare in this disease [24].

## *3.4. Urogenital tract*

**3.4.1. Renal tumors.** In the early studies of oncogene amplification in human tumors, there was some evidence that c-*erb*B2 was occasionally amplified in renal cell carcinoma [26,28]. However, more focused studies of this particular disease have shown that 11 out of 13 renal cell carcinomas and one Wilm's tumor examined by Northern blot analysis showed decreased expression of c-*erb*B2 relative to normal kidney [118]. Further comparison with epidermal growth factor receptor expression revealed an inverse relationship, with 73% of renal cell carcinoma overexpressing EGF receptor and 93% having reduced c-*erb*B2 mRNA levels [119]. In no cases was there evidence of gene rearrangement or amplification. It seems unlikely, therefore, that c-*erb*B2 plays a significant role in the development of renal cell carcinoma [119].

**3.4.2. Bladder cancer.** The reported expression of c-*erb*B2 in transitional cell carcinoma of the bladder varies from 2% [81] to as high as 74% [120], with an intermediate value of 30% reported by Gullick et al. [121]. This is

rarely associated with gene amplification; in the series giving 74% positivity by antibody staining, only 8% of tumors analyzed by Southern blot gave evidence of increased copy number [122]. Moriyama et al. recently published data linking c-*erb*B2 with prognostic indicators in bladder cancer [123] in which 37% of 54 human bladder cancers were c-*erb*B2 positive by immunocytochemistry. When stratified into histological grade, there was a strong association with less differentiated tumors: 15% positive in G1, 26% in G2, and 69% in G3. In terms of invasiveness, 88% of invasive tumors vs. 20% of noninvasive had positive membrane staining ($p < .01$). As yet there are no follow-up data on c-*erb*B2 positive cases to assess its role as a prognostic indicator in bladder cancer, but it seems likely that it does have a role in transitional cell carcinogenesis.

There is no reported overexpression of p185 in prostatic cancer to date [81].

### *3.5. Lung cancer*

Normal ciliated bronchial epithelium expresses low levels of $p185^{neu}$. Elevated levels have not been found in small-cell lung cancer. However, amongst non-small-cell lung cancer increased expression above normal levels is seen in up to 30% [124–126] of primary tumors.

In the study by Weiner et al., 4 of 12 adenocarcinomas, 1 of 2 squamous carcinomas, 2 of 3 large-cell carcinomas, and 7 of 24 of all specimens were positive for c-*erb*B2 membrane staining [125]. More recently the same group has extended its study to 55 non-small-cell primary lung tumors. A similar proportion have increased membrane staining by immunocytochemistry. In the adenocarcinoma patients such staining was associated with a significantly reduced overall survival and was an independent predictor of survival, even after correction for the stage of disease [126]. Positive staining in squamous cell carcinoma had no demonstrable predictive value.

### *3.6. Endocrine tumors*

In the initial studies on a small series of a range of human tumors, there was no evidence for c-*erb*B2 amplification or overexpression in thyroid tumors [127], which has subsequently been confirmed [22]. Other endocrine tumors that have evidence for c-*erb*B2 overexpression include pulmonary atypical carcinoid (6 of 15), pheochromocytoma (13 of 27), and insulinoma (4 of 17) [127].

### *3.7. Other tumors*

In Yokota et al.'s original paper no evidence for c-*erb*B2 amplification was found in sarcomas, leukemias, or lymphomas [28], nor was amplification

reported in esophageal or head-and-neck tumors, although only small numbers were studied. This report has not yet been contradicted. Similarly there is no evidence to indicate c-*erb*B2 overexpression in brain tumors [23]. One report has shown c-*erb*B2 membrane staining in the squamous cell layer of the surface epidermis of squamous cell carcinoma but little evidence for overexpression in basal cell carcinoma [128].

## 4. Summary

There is no evidence for activation of c-*erb*B2 by mutation in human cancer. Gene rearrangements are observed at low frequency, but there are a proportion of human cancers that are associated with c-*erb*B2 gene amplification and membrane protein overexpression. The human cancers so affected are adenocarcinomas of the breast, ovary, stomach, and bladder, with up to 20% of primary lesions exhibiting either increased gene copy number and/or excess membrane staining. The c-*erb*B2 protein on these tumors could be used as a therapeutic target, as in monoclonal antibody targetted therapy already being assessed in c-*erb*B2 positive breast cancer. Other possible therapeutic strategies include the development of tyrosine kinase inhibitors or ligand antagonists.

## Acknowledgments

We would like to thank Zoe Redley for help in preparing this manuscript.

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# 9. Clinical significance of *erb*B2 protein overexpression

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## 1. Introduction

Originally described as a homologue of the EGF receptor gene amplified in breast cancer [1], *erb*B-2 protein is now thought to be a cell membrane receptor for a newly described ligand-gp30 [2]. There is some confusion in the literature about the terminology for *erb*B-2. In brief, *erb*B-2, c-*erb*B-2, and HER-2 refer to the same human gene residing in the long arm of the chromosome 17, which has homology to human EGF receptor gene (c-*erb*B) [3]. *erb*B-2 protein, p185*erb*B-2, or p185HER-2 refer to the transmembrane receptor protein translated from the *erb*B-2 gene, which has a molecular weight of 185 kDa. c-*neu* refers to the rat counterpart of the *erb*B-2 gene [4].

Carcinomas from many organs have frequent overexpression of *erb*B-2 protein. These include carcinomas of the breast [5], lung [6], ovary [7], stomach [8], pancreas [9], and endometrium [10]. The overexpression of *erb*B-2 protein is usually accompanied by amplification of the gene. However, in about 10% of the tumors that overexpress *erb*B-2 protein, no amplification is found, as in the T-47-D breast cancer cell line [11].

In this review, we will concentrate mainly on the clinical importance of *erb*B-2 protein overexpression in human cancers.

## 2. Function of *erb*B-2

As shown in Figure 1, *erb*B-2 protein is thought to be composed of three domains — the extracellular ligand binding domain, transmembrane domain, and intracellular tyrosine kinase domain [3]. Since the ligand was identified only recently, signal transduction through *erb*B-2 was still poorly understood. However, studies utilizing hybrid receptors (the extracellular domain of EGF receptor/intracellular domain of *erb*B-2, see Chapter 11) have demonstrated that stimulation of *erb*B-2 may result in activation of early-response genes, such as *jun* and *fos* [12].

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

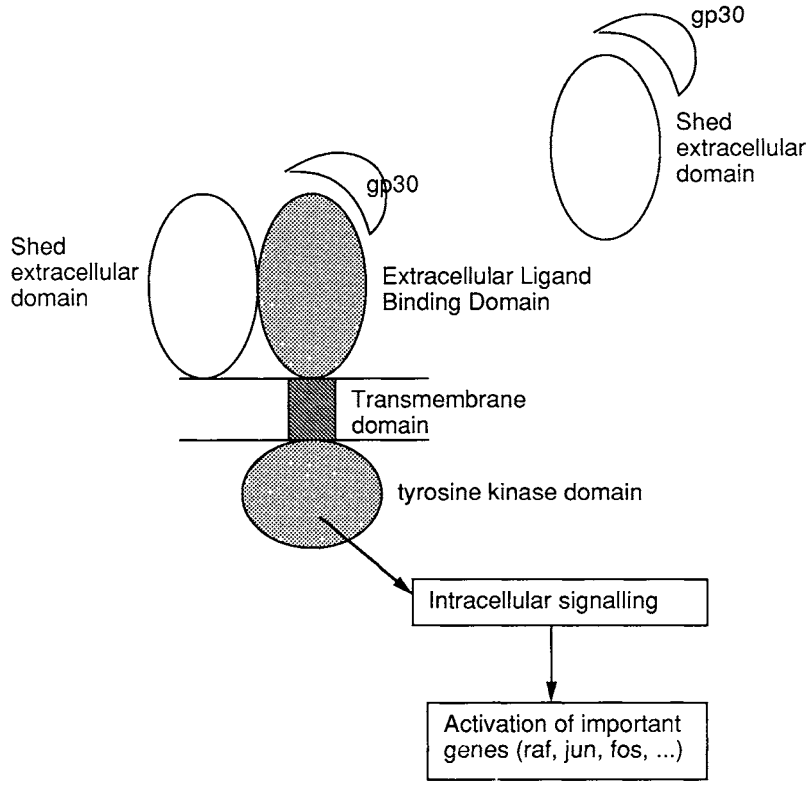



*Figure 1.* Schematic representation of the postulated domains of erbB-2 protein and its interaction with ligand and ligand-like molecules; shed extracellular domain may directly interact with the receptor or may block the binding of the gp30 ligand to the receptor. Heterodimerization with the other receptor may also be important in the regulation of signal transduction as well as the phosphatases.

## 3. Ligand and ligand-like activities for *erb*B-2

Recently a putative ligand for *erb*B-2 has been described by Lupu et al. [2]. This gp30 ligand activates phosphorylation of both EGF receptor (EGF-R) and *erb*B-2. Although it is known that EGF-R can crossphosphorylate *erb*B-2 [13,14], gp30 showed both biological and biochemical effects on cells with *erb*B-2 overexpression but without EGF-R [2]. Thus gp30 seems to act as a ligand for *erb*B-2. Intriguingly, gp30 ligand did not show any demonstrable effect on cells with normal levels of *erb*B-2 [2]. The shed extracellular domain of *erb*B-2 represents another class of ligand-like activity, which might play a role in vivo [15]. Theoretically such a shed domain can directly interact with membrane-bound *erb*B-2 protein, with or without

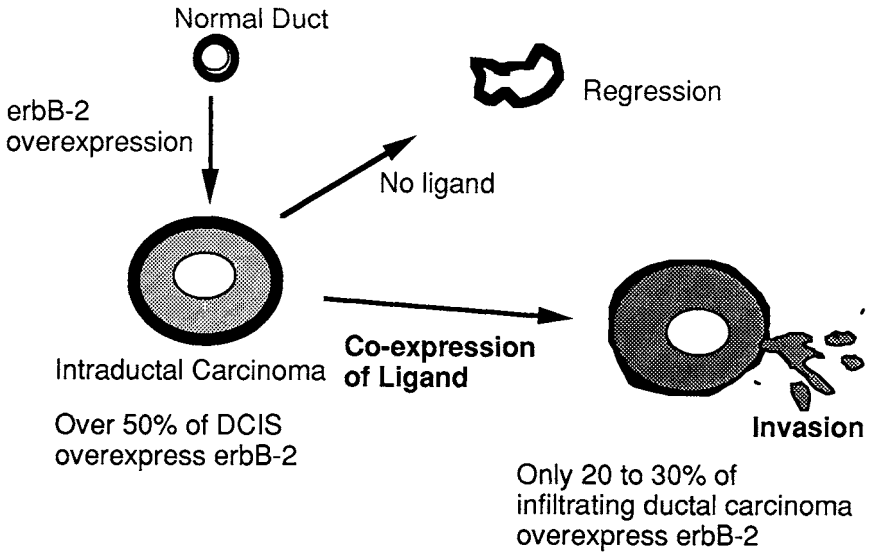



*Figure 2.* Hypothesized role of the ligand in the diseasse progression in breast cancer; tumor cells that express both the receptor and the ligand would result in infiltrating ductal carcinoma. Some tumors may begin to express both and bypass the intraductal stage, thus resulting in infiltrating ductal carcinoma without the intraductal component.

biological effects, or may even block the interaction between gp30 ligand and the receptor (see Figure 1). The shed extracellular domain may even be useful for patient follow-up during and after treatment. Thus it is apparent that the *erb*B-2 signal transduction pathway presents an extremely complex interaction of receptors, ligands, and ligand-like molecules. We are still far from understanding the role of each component.

As will be discussed later, the incidence of *erb*B-2 overexpression is much higher in intraductal breast cancer compared to infiltrating ductal carcinoma [16]. We have frequently seen cases in which only the intraductal component is positive, whereas the infiltrating component is negative. Based on these findings, we propose an '*erb*B-2 ligand invasion' hypothesis, as shown in Figure 2. According to this hypothesis, overexpression of *erb*B-2 is a fairly early event in the progression and does not result in an invasive phenotype on its own. It is believed that about one third of the intraductal cancers actually progress to infiltrating ductal carcinoma. Only when the gp30 ligand is coexpressed could *erb*B-2-mediated cell invasion be initiated and tumor become invasive. Generation of cDNA probe and antibodies for the ligand(s) will be required to test such a hypothesis. Of course it should be remembered that not all tumors with *erb*B-2 overexpression have intraductal components. In such tumors, *erb*B-2 and the ligand might have been expressed together at an early stage, according to this model.

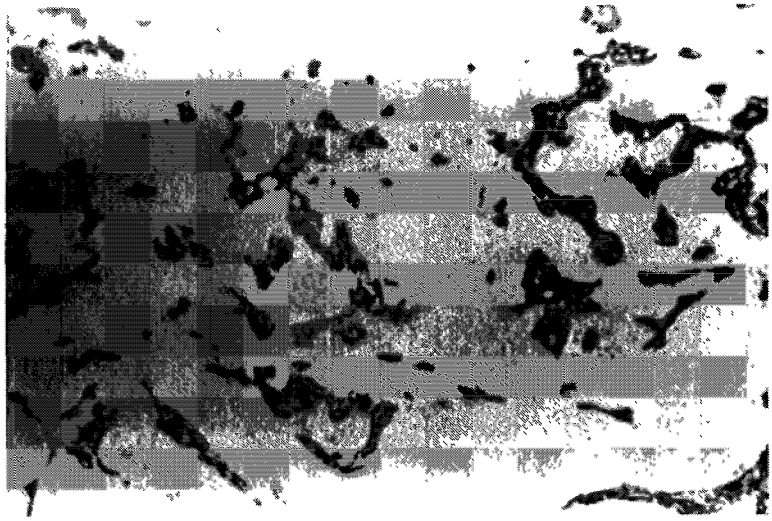
## 4. Role of the transmembrane domain in transformation

Although the transforming *neu* gene (the rat homologue of the human *erb*B-2) has a point mutation in the transmembrane domain [4], no such transforming mutations have been found yet for the human *erb*B-2 (17). While this may be partly due to the technical difficulties involved in detecting mutations in amplified gene, overexpression of the *erb*B-2 protein alone without any mutation have been shown to transform NIH-3T3 cells [18]. However, possibilities for mutations are not completely excluded.

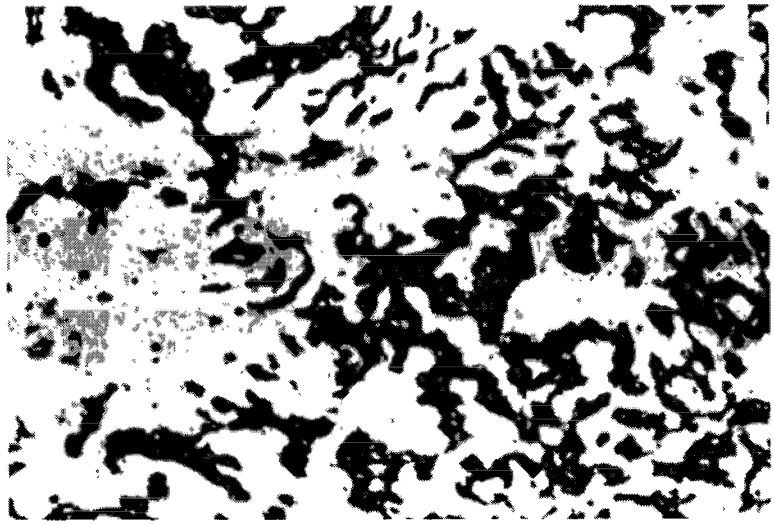
## 5. Immunostaining for *erb*B-2 protein

Although Slamon and coworkers were the first to show the potential clinical importance of *erb*B-2 gene amplification [19], the development of immunostaining assays that allowed the detection of *erb*B-2 overexpression in paraffin-embedded sections has been the major breakthrough in studying the clinical importance of *erb*B-2 [5]. Immunostaining assays detect only the tumor cells with overexpression of *erb*B-2 protein, not the cells with normal levels of *erb*B-2. The breast-cancer cell lines MCF-7 or MDA-MB-468 are examples of tumor cells expressing only the normal (unamplified) amount of *erb*B-2 protein. Shown in Figure 3b is an example of a breast cancer with *erb*B-2 protein overexpression. Note the strong membrane staining of all tumor cells in the section. Usually all tumor cells stain positive when a given tumor is stained positive, including both in situ and invasive components, as well as the metastatic site. Many studies have shown a paradoxically higher incidence of *erb*B-2 overexpression in intraductal cancer of the breast [20]. This disease is believed to be in situ and a preinvasive cancer. We have never observed a case in which the invasive component is positive when the in situ component is negative. However, we have seen cases in which the in situ component is positive but the invasive component is negative, or in which the primary tumor is positive but the metastatic site is negative. Thus overexpression of *erb*B-2 seems to be an early event in the progression of breast cancer.

Although some investigators have questioned the sensitivity of the immunostaining assay for paraffin-embedded sections, we have found that a cocktail preparation of antibodies directed against the extracellular and intracellular domains (pAb-1 plus mAb-1, Triton Biosciences) can be used to increase the sensitivity of the assay almost threefold compared to polyclonal antibody against the cytoplasmic domain (pAb-1) used alone. Figure 4 shows an example of cases that were strongly stained with the cocktail preparation (Figure 4a) but did not show staining at all with pAb-1 alone (Figure 4b). Furthermore, even in cases that showed heterogenous staining by one antibody (an example is shown in Figure 3a), the cocktail antibody stained all tumor cells in the section (Figure 3b), thus improving the objec-

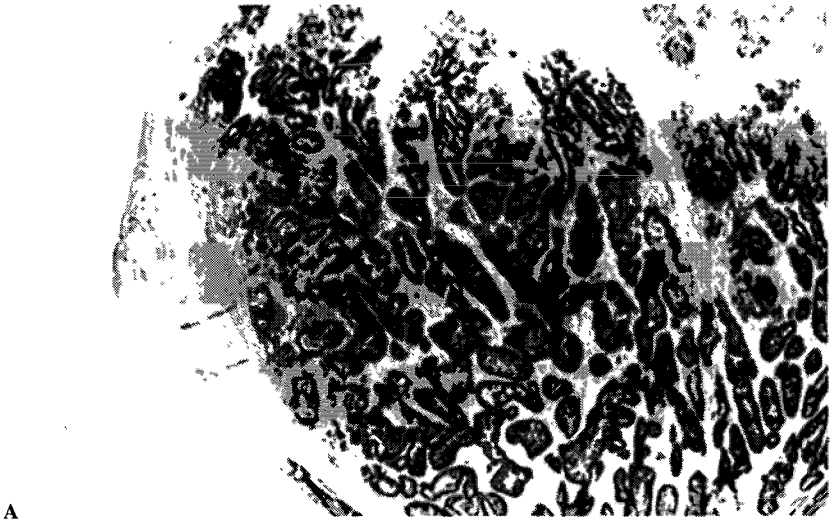


A



B

*Figure 3.* Enhancement of immunostaining of monoclonal antibody by the addition of polyclonal antiserum. **A**: Staining with monoclonal antibody (mAb-1) alone resulted in heterogenous staining due to the failure to recognize the epitope in the center of the formalin-fixed and paraffin-embedded tissue section (20× magnification). **B**: Addition of the polyclonal antiserum (pAb-1) resulted in homogenous staining of all tumor cells in the section.



A

B

*Figure 4.* Comparison of immunostaining by the cocktail antibody (mAb-1 plus pAb-1) vs. the polyclonal antiserum (pAb-1), which has been commonly used in the published studies. **A**: The cocktail antibody stains all tumor cells in this case of gastric adenocarcinoma (20× magnification). **B**: Polyclonal antiserum fails to stain any tumor cells in the same section.

tivity of the interpretation of the assay result. The immunostaining assay is now generally accepted as the method of choice for the detection of *erb*B-2 overexpression in the clinical tumor samples [21]. Obviously, this method has many practical advantages over DNA, RNA, or protein blotting techniques. Recent progress in automatic immunohistochemistry promises a bright future for the routine clinical application of this assay.

## 6. Clinical importance of *erb*B-2 protein overexpression

Among the tumors that overexpress *erb*B-2, breast cancer has been most extensively examined. Slamon is responsible for many of the initial studies. By screening a variety of human tumor samples for the existence of possible gene amplification or overexpression of almost all known oncogenes, Slamon has successfully shown that *erb*B-2 gene amplification is frequent in breast cancer and is a potentially important prognostic indicator in stage 2 breast cancer. However, much of the clinical interest in *erb*B-2 was mainly due to the hope that one might be able to use the assay to identify the patients with node-negative breast cancer who are at high risk and should be treated with adjuvant systemic treatments. Indeed many studies, including our own, have shown the prognostic value of *erb*B-2 overexpression/gene amplification in breast cancer [reviewed in 21]. Patients with *erb*B-2-positive tumors are probably at higher risk and should be treated with adjuvant therapy. This may be especially true in the case of those with node-negative breast cancer with good nuclear grade or ER-positive status, who otherwise would not be candidates for chemotherapy [5,22]. However, what has been emerging from recent studies is the realization that, in addition to its role as an indicator for poor prognosis after surgical treatment without adjuvant therapy [23], *erb*B-2 is an indicator for poor prognosis even for patients treated with adjuvant regimens — PF (L-PAM, 5-FU) [5], CMF (cytoxan, methotrexate, 5-FU), CMFP [24], or tamoxifen [25]. There is preliminary evidence that suggests *erb*B-2 overexpression correlates with a lack of response to at least some of these regimens [26,27]. If *erb*B-2-positive tumors still have a poor prognosis when treated, other approaches will have to be employed. For the latter goal, in vitro studies have been carried out exploring monoclonal antibodies against *erb*B-2 protein [28 and Chapter 10] and antisense oligodeoxynucleotides directed against *erb*B-2 mRNA [29]. At the same time, alternate conventional chemotherapeutic regimens that might be effective for *erb*B-2-positive tumors should be explored. Insight into this possibility can be accomplished by retrospective screening of the completed clinical trials comparing different adjuvant regimens. We have observed that breast cancer cell lines with *erb*B-2 overexpression tend to be resistant in vitro to suprapharmacological doses of 5-FU, L-PAM, and mitomycin C, but not to anthracyclines (doxorubicin and mitoxantrone) in a drug resistance assay [30]. Thus we hypothesize that *erb*B-2-positive tumors

should respond as well to doxorubicin-containing regimen as *erb*B-2-negative tumors. If the latter hypothesis is true, *erb*B-2 determination would not be a prognostic indicator for tumors treated with doxorubicin-containing regimens, while being a prognostic indicator for tumors treated with surgery only or nondoxorubicin adjuvant chemotherapeutic regimens. Such studies are in progress for breast cancer in collaboration with the NSABP (National Surgical Adjuvant Breast Project) — NSABP protocols B-11 and B-12 — in which more than 1400 node-positive patients were randomized to receive either PF or PAF. While such studies are in progress we have also examined this hypothesis in gastric cancer. Although included in the earliest screening studies [31,32], *erb*B-2 has not been studied extensively in gastric cancer. Most of the studies examining gene amplification observed amplification only in well-differentiated (or tubular) adenocarcinoma but not in poorly differentiated adenocarcinomas [33,34]. More recently, studies using immunohistochemistry have shown that overexpression is not confined to tubular cancer, and the incidence is between 10 and 50%, depending on the study [8,35,36]. There are only two studies in the literature that have examined the effect of *erb*B-2 overexpression on patient outcome. Of these the study by Yonemura and coworkers deserves particular attention [8]. They reported that *erb*B-2 was found to be an independent indicator for poor prognosis for patients treated with adjuvant regimes — MF (mitomycin C plus oral 5-FU) or oral 5-FU alone. A gastric cancer cell line that overexpresses the *erb*B-2 protein (NCI-N87) is also resistant to 5-FU [37]. In a retrospective study with small number of cases, we found that *erb*B-2 is a prognostic indicator for patients treated with surgery alone, but not for patients treated with a doxorubicin-containing regimen [38]. Together with the data by Yonemura et al., the data suggest that tumors with *erb*B-2 overexpression may be resistant to an MF regimen but not to doxorubicin-containing regimens and that further studies in this direction are required.

## 7. Therapeutic importance of *erb*B-2

Whether *erb*B-2 overexpression is a causative variable or an associated variable for a poor drug response is a clinically important question. This is true because if overexpression of *erb*B-2 is causative, inhibition of *erb*B-2 may result in reversion of the drug-resistant phenotype. Hancock and coworkers have shown that an anitbody against *erb*B-2 can enhance the cytocidal effect of cisplatin on ovarian cancer cell line with *erb*B-2 overexpression [39]. Benz and coworkers have also shown that transfection of *erb*B-2 into MCF-7 cells results in a decrease in sensitivity to tamoxifen and cisplatin [27]. Thus it seems possible that *erb*B-2 is at least associated with, if not responsible for, decreased sensitivity to various chemotherapeutic drugs, which can be overcome by interfering with the *erb*B-2 signal transduction pathway. This suggests the possibility that therapeutic approaches can be

developed by combining conventional drugs plus reagents that will block *erb*B-2, such as antibodies or antisense oligodeoxynucleotides.

The data by Bacus and coworkers on *erb*B-2 and ploidy deserves attention from the therapeutic point of view [41]. They showed in small number of breast cancer cases that *erb*B-2 overexpression is highly associated with hypertetraploid DNA content and a low proliferation rate. If these data hold true, then cell-cycle-specific drugs, as suggested by Clark and coworkers, may not be an ideal treatment strategy [42]. However, there are many larger studies that contradict these results, although at least one study showed that *erb*B-2 overexpression is associated with hypertetraploidy [25]. This question should be more carefully addressed in the future studies in light of the therapeutic viewpoint.

Regardless of its relationship with the chemotherapeutic responses, *erb*B-2 seem to be an extremely attractive target for experimental therapeutics. Since (1) only the cells with *erb*B-2 overexpression respond to the growth-inhibitory effects of monoclonal antibodies or gp30 ligand, and (2) when a given tumor is positive for *erb*B-2 overexpression all tumor cells overexpress *erb*B-2, one can easily visualize the possibility of the clinical application of such molecules.

## 8. Future directions

During the past several years we have learned much about the clinical importance of *erb*B-2 protein overexpression. However, we do not know the cellular and biochemical mechanisms responsible for such clinical behavior. As such mechanisms are elucidated, we will be able to take adventage of the situation and use overexpressed *erb*B-2 as a therapeutic target for novel agents, as well as a guide for custom-tailored therapies for individual tumors.

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# 10. Anti-p185HER2 monoclonal antibodies: Biological properties and potential for immunotherapy

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## 1. Introduction

It is by now clear that the pathogenesis of human cancer involves the aberrant functioning of the products of oncogenes. In breast and other cancers, the HER2 protooncogene appears to play an important role in the development and progression of these diseases. As such, it represents a potential focus for therapeutic intervention.

One way to do this is to derive monoclonal antibodies directed against p185HER2, the HER2 or c-*erb*B-2 gene product. Unlike other attempts at immunotherapy using antibodies raised against various tumor-associated antigens, this approach seeks to use antibodies to directly modulate tumor behavior. The treatment of cancer with monoclonal antibodies generally relies upon host immunologic mechanisms to purge the tumor cells recognized by antibody; otherwise, it relies upon linking the antibody with radionuclides, toxins, or other cytotoxic moieties. However, when a crucial contributor to the neoplastic phenotype (such as a growth factor receptor) is targeted, antibody binding per se may produce profound biological effects.

In this chapter we will first present a very brief overview of the HER2 protooncogene to illustrate why it is a compelling target for immunotherapy. (For a more comprehensive review of HER2 itself, the reader is referred to other chapters in this volume.) We will then discuss our studies in the development and characterization of monoclonal antibodies directed against p185HER2, including our findings on the effects of these antibodies upon tumor cell growth in both in vitro and in vivo model systems. We will also describe the construction through genetic engineering of humanized versions of an anti-p185HER2 monoclonal antibody; such antibodies may have advantages as therapeutic agents. Finally, we will summarize our experience in preparing one of the murine monoclonal antibodies for use in the clinic.

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

## 2. The HER2 protooncogene

### *2.1. Discovery and characterization of HER2*

The rat oncogene *neu*, like many oncogenes, was identified by virtue of its ability to transform NIH3T3 cells in transfection assays. In this case, the transfected DNA was derived from neuroblastomas that had arisen in rats exposed in utero to the carcinogen ethylnitrosourea [1]. The transforming gene was isolated and designated *neu* in reference to its origin from rat neuroblastomas. Characterization of this gene revealed that it encodes a 185,000 MW membrane-bound glycoprotein with the structure of a growth factor receptor tyrosine kinase. Like other members of this family, it consists of an extracellular ligand-binding domain (for an as yet unidentified ligand), a transmembrane domain, and an intracellular tyrosine kinase domain. Furthermore, this receptor has extensive homology with the human receptor for epidermal growth factor (EGF) [2].

Additional studies of the *neu* oncogene provided insight into *neu*-mediated transformation. The dominant transforming gene obtained from rat neuroblastoma DNA, when compared with its nontransforming counterpart from normal rat cells (the *neu* protooncogene), was found to possess an activating point mutation (adenine to thymidine) within its transmembrane domain, yielding a valine to glutamic acid substitution at position 664 [3]. This mutation leads to a constitutive increase in the tyrosine kinase activity of the *neu* gene product [4], and ultimately to the malignant phenotype.

The HER2 gene was identified by screening human genomic and cDNA libraries for homologues of the gene for the EGF receptor [5–7]. The HER2 gene, in fact, proved to be the human equivalent of the rat *neu* protooncogene. This gene has been referred to as HER2 or c-*erb*B-2 because of its homology with the *h*uman *e*pidermal growth factor *r*eceptor gene (HER1 or c-*erb*B-1); it is sometimes referred to as *neu* or HER2/*neu* as well. In this review, we will adhere to the designation HER2 for the gene, and p185HER2 for the oncoprotein gene product.

### *2.2. HER2 and human malignant diseases*

Unlike the rat *neu* protooncogene, in which an activated form of the gene arises from point mutation, the HER2 protooncogene does not appear to have an activated mutant form. However, the gene was found to be amplified in the MKN7 cell line derived from human gastric cancer and in the MAC117 cell line derived from human breast cancer [6,8]. Further studies with other cell lines from human tumors have similarly shown HER2 amplification to be a frequent occurrence, particularly in cell lines that are adenocarcinomas in their histologic derivation. This contrasts with the related EGF receptor, which is often amplified in cell lines from squamous

cell carcinomas, but rarely in cell lines from adenocarcinomas. In addition to amplification of the gene at the DNA level, some human breast cancer cell lines have been found to have HER2 overexpression at the RNA level, as assessed by Northern blot analysis, without concomitant gene amplification [9].

When DNA from primary tumors of human breast cancer were studied by Southern blot analysis, about 30% of these tumor samples were found to possess amplifications of the HER2 gene [10]. Furthermore, in those patients with axillary lymph-node-positive breast cancers, the presence of HER2 amplification correlated with significantly worsened clinical outcomes: HER2 amplification predicted decreased overall survival and shortened time to relapse, and was a strong and independent prognosticator by multivariate analysis. Later, these findings of frequent HER2 amplification and its clinical impact were complemented by the demonstration in human tumors of HER2 overexpression at the RNA level by Northern blots, and at the protein level by Western blots and by immunohistochemistry; HER2 overexpression as assessed by these methods was similarly predictive of poor outcome in patients with node-positive breast cancer [11,12]. Recently, HER2 overexpression has been found to have prognostic significance in certain subgroups of node-negative breast cancer patients as well [13], and possibly in node-negative patients as a whole [14].

In addition to breast cancer, HER2 amplification and/or overexpression has now been demonstrated in a number of other human malignant diseases, again typically but not exclusively in those that are adenocarcinomas by histologic origin. Neoplasms in which HER2 amplification and/or overexpression occurs and has been correlated with worsened prognosis include ovarian cancer [11,15], endometrial cancer [16,17], gastric cancer [18], and adenocarcinoma of the lung [19]. In addition, HER2 amplification and/or overexpression has also been observed in salivary gland adenocarcinoma [5,20], squamous cell caricinoma of the lung [19,21], colon cancer [22,23], transitional cell carcinoma of the bladder [24–26], thyroid cancer [27], and a single case of diffuse large-cell non-Hodgkin's lymphoma [28].

### *2.3. The role of HER2 in the biology of human cancer*

The fact that increased expression of the HER2 protooncogene is associated with poor prognosis in breast and other cancers suggests that its gene product, p185HER2, may play an important role in determining the biological behavior of these tumors. That HER2 overexpression does indeed lead to aggressive tumor behavior, and is not merely a marker of this phenotype, is supported by a several lines of experimental evidence. First, when transfected and overexpressed in NIH3T3 cells, HER2 is transforming: These cells undergo morphologic change, form foci in culture, form colonies in soft agar, and grow aggressively in nude mice [29,30]. Studies with transgenic mice further demonstrate the tumorigenicity of HER2 over-

expression. When the rat *neu* oncogene with its activating point mutation (see above) was expressed in transgenic mice under the control of the mouse mammary tumor virus promoter, the transgenic mice consistently developed mammary carcinoma [31]. When the human HER2 gene was expressed in transgenic mice, a variety of tumors were induced [32]. In this experiment, transgenic mice were constructed with either the normal HER2 proto-oncogene or a mutated HER2 gene containing the same activating point mutation as that in the rat *neu* gene; both alleles induced adenocarcinomas, particularly of the lung and lacrimal gland, as well as several types of lymphoma.

In addition to being tumorigenic in these model systems, HER2 overexpression may also contribute to tumor resistance to host immune defenses, thus further promoting tumor growth [for review, see 33]. NIH3T3 cells expressing high levels of transfected HER2 acquired resistance to killing by activated macrophages or to recombinant TNF-α; similarly, breast cancer cell lines that overexpress p185HER2 tended to be more resistant to killing by these effectors than were cell lines that do not [34]. A correlation between HER2 overexpression and resistance to TNF, as well as resistance to lymphokine-activated killer (LAK) cells, was also observed in some ovarian cancer cell lines [35].

The precise mechanisms by which overexpression of p185HER2 leads to aggressive tumor behavior are not yet understood. As previously discussed, the close homology with the EGF receptor makes it plausible that p185HER2 functions as a receptor tyrosine kinase that is activated by ligand binding. Alternatively, p185HER2 may function autonomously when overexpressed [36].

Because of the compelling evidence that p185HER2 is intimately involved in the pathogenesis of breast and other human cancers, we have sought to generate monoclonal antibodies directed against p185HER2 for use as reagents in further studies of HER2 and as potential therapeutic agents in the treatment of these diseases. Such antibodies should bind preferentially to p185-overexpressing tumor cells and not to normal tissue. The limited level of expression of p185HER2 in normal cells and tissues suggests that this is a feasible approach [37]. Furthermore, just as antireceptor antibodies in other systems can sometimes evoke profound biological responses, anti-p185HER2 MAbs might antagonize the function of p185HER2 and thereby exert a powerful effect on tumor growth.

## 3. Anti-p185HER2 monoclonal antibodies

### *3.1. Production of murine monoclonal antibodies specifically directed against p185HER2*

Monoclonal antibodies directed against the extracellular domain of p185HER2, the HER2 or c-*erb*B-2 gene product, were generated in mice

[38]. The antigen source used was an NIH3T3 cell line, designated NIH3T3/HER-2-3-400, in which HER2 had been stably transfected, amplified, and expressed at very high levels. BALB/c mice were immunized with these cells, and were also boosted with a p185HER2 preparation obtained from membrane extracts of this cell line; splenocytes from the immunized mice were then fused with mouse myeloma cells to yield hybridomas. Hybridoma supernatants were screened with an ELISA assay to detect MAb binding to immobilized p185HER2. This screening identified ten monoclonal antibodies that displayed high affinity for p185HER2 and, importantly, no significant binding to the closely related EGF receptor (Table 1).

The specificity of these MAbs was confirmed by a number of complementary approaches. First, the MAbs were used in immunoprecipitations of autophosphorylated, $^{32}$P-labeled p185HER2 or EGFR: Only p185HER2 and not the 170-kDa EGFR was precipitated. Next, immunoprecipitations were performed using cell lysates from $^{35}$S-metabolically labeled cells. Again, only p185HER2 was precipitated (Figure 1A). Finally, FACS analysis of MAb binding was done using the breast cancer cell line SK-BR-3, which greatly overexpresses p185HER2, or the squamous cell cancer cell line A431, which overexpresses EGFR. Eight of the 10 MAbs bound SK-BR-3 cells with high RMF values, while none of the MAbs showed

*Table 1.* Summary of monoclonal antibodies described

| MAb | Isotype | EGFR ELISA | p185$^{HER2}$ ELISA | EGFR RIP | p185$^{HER2}$ RIP | Epitope | FACS |
|---|---|---|---|---|---|---|---|
| 4D5 | IgG1,k | – | ++ | – | ++ | I(p/c) | +++ |
| 2C4 | IgG1,k | – | +++ | – | ++ | F(p/c) | +++ |
| 2H11 | IgG2a,k | – | + | – | ++ | H(p/c) | ++ |
| 3E8 | IgG2a,k | – | + | – | +++ | H(p/c) | +++ |
| 3H4 | IgG1,k | – | + | – | + | I(p) | + |
| SB8 | IgG1,k | – | + | – | ++ | nd(p) | + |
| 6E9 | IgG1,k | – | ++ | – | + | nd(p) | – |
| 7C2 | IgG1,k | – | ++ | – | ++ | G(p) | +++ |
| 7D3 | IgG1,k | – | ++ | – | ++ | F(p/c) | +++ |
| 7F3 | IgG1,k | – | +++ | – | +++ | G/F(p/c) | +++ |

ELISA data columns — summary of O.D. 492 nm: (–), <0.1; (+), 0.11–0.50; (++), 0.51–1.0; (+++), >1.0.

RIP data columns — summary of autoradiography from immunoprecipitations: (–), bands equal to negative control; (+), weak bands but darker than negative control; (++), moderately exposed bands; (+++), strongly exposed bands.

Epitope data columns: letters were assigned to represent individual epitopes A through I (nd = not done). MAbs were considered to share an epitope if each blocked binding of the other by 50% or greater in comparison to an irrelevant MAb control. The epitope composition recognized by immunoprecipitations with each MAb from tunicamycin-treated cells is shown. The letters p, c, or p/c in parentheses indicate that the monoclonal antibody binds only to the polypeptide (p), the carbohydrate (c), or both (p/c) moieties in the extracellular domain of p185$^{HER2}$.

FACS data column: fluorescense staining of SK-BR-3 cells by the anti-p185$^{HER2}$ monoclona antibodies. (–), MAbs equal to the negative control MAbs; (+), one- to nine-fold higher than the negative controls; (++), 10- to 99-fold higher than the negative controls; (+++), >100-fold higher than the negative controls.

significant binding to A431 cells (a sample FACS histogram for muMAb 4D5 binding is shown in Figure 1B). Two MAbs (5B8 and 6E9) failed to show high relative mean fluorescence (RMF) for binding to either cell line, presumably because these MAbs bind to epitopes close to the transmembrane domain of p185HER2, which may not be readily accessible to antibody in intact SK-BR-3 cells. These specificity data are summarized in Table 1, which also shows putative epitope assignments based on MAb competition assays.

### *3.2. Antiproliferative effects on cultured cell lines*

To investigate the biological effects of these anti-p185HER2 MAbs on tumor cells, six of the MAbs (muMAbs 4D5, 3H4, 2C4, 7F3, 7C2, and 6E9) were tested for their effect on in vitro tumor cell growth. With the SK-BR-3 cell line, the MAbs produced markedly different effects on growth, ranging

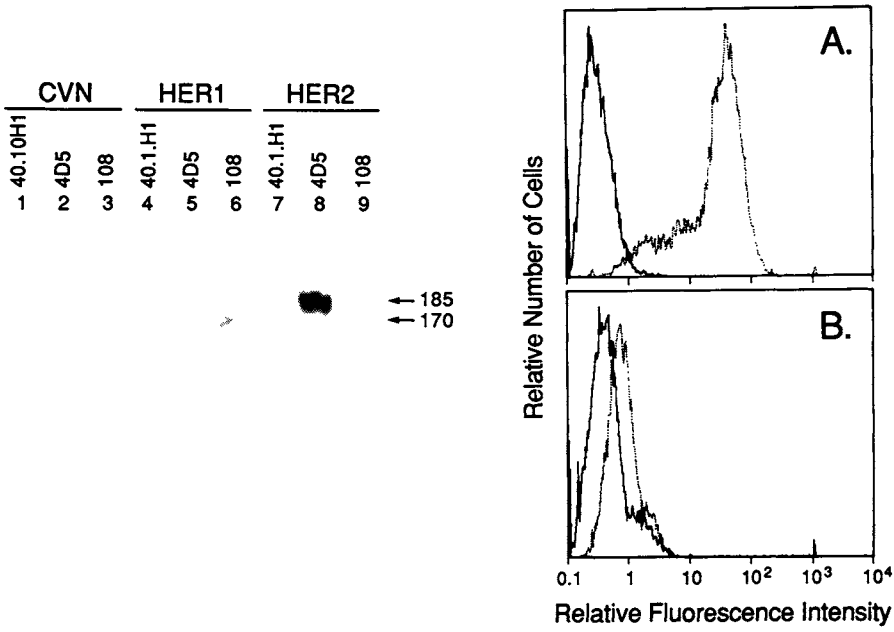


*Figure 1.* MAb 4D5 does not crossreact with EGFR. A: Immunoprecipitation of $^{35}S$-metabolically labeled NIH3T3 cells transfected by control plasmid (CVN), by a plasmid encoding the EGFR (HER1), or a plasmid encoding p185HER2 (HER2). MAb 40.1.H1 is directed against hepatitis B surface antigen (lanes 1,4,7); MAb 4D5 is directed against p185HER2 (lanes 2,5,8); MAb 108 is directed against EGFR. B: Fluorescence-activated cell sorter histograms of MAb 40.1H1 (solid lines) or MAb 4D5 (broken lines) reacted with SK-BR-3 tumor cells (approximately $2 \times 10^6$ p185HER2 molecules per cell surface; upper panel); or the same MAbs reacted with the A431 squamous carcinoma cell line (approximately $2 \times 10^6$ EGFR per cell surface; lower panel).

from no effect to strong inhibition (Table 2). Notably, muMAb 4D5 was most effective at inhibiting cell proliferation (to 33% of control growth). These MAbs were then further tested against a battery of cell lines: These included a nonmalignant breast epithelial cell line (184), eight breast cancer cell lines, and an ovarian cancer cell line (SK-OV-3). These lines comprise a wide spectrum of target antigen (i.e., p185HER2) expression. As depicted in Table 2, the six anti-p185HER2 MAbs inhibited tumor cell growth to varying degrees, depending on which MAb and which cell line were used. The MAbs 4D5, 3H4, 2C4, and 7F3 generally inhibited tumor cell growth in cell lines overexpressing p185HER2, but showed a negligible effect on the nonmalignant cells (184) and on the malignant cell lines expressing low levels of p185HER2. Moreover, for each of these MAbs, the inhibitory effect on cell growth largely correlated with the quantity of p185HER2 present at the cell surface. In contrast, the MAbs 7C2 and 6E9 demonstrated minimal growth-inhibiting activity with any cell line, and modestly stimulated growth in some cell lines. Taken together, these data demonstrate that some anti-p185HER2 MAbs can inhibit the growth of cultured tumor cell lines that overexpress p185HER2.

Among the antibodies that were able to retard tumor cell growth, muMAb 4D5 appeared to be the most effective. With the p185HER2-overexpressing cell lines MDA-MB-453, MDA-MB-361, SK-BR-3, and SK-OV-3, the greatest growth inhibition was obtained with muMAb 4D5; while with the remaining overexpressing cell lines (MDA-MB-175 and BT-474), muMAb 4D5 was among the best of the MAbs in producing

*Table 2.* Inhibition of human tumor cell growth by anti-p185HER2 monoclonal antibodies

| Cell line | Relative p185HER2 expression[a] | Relative cell proliferation (% of control)[b] | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4D5 | 3H4 | 2C4 | 7F3 | 7C2 | 6E9 |
| 184 | 1 | 116 | 114 | 109 | 116 | 117 | 103 |
| MCF7 | 1 | 101 | 113 | 100 | 111 | 112 | 105 |
| MDA-MB-231 | 1 | 91 | 100 | 93 | 98 | 104 | 103 |
| ZR-75-1 | 3 | 102 | 105 | 99 | 97 | 108 | 97 |
| MDA-MB-175 | 5 | 62 | 77 | 29 | 48 | 87 | 96 |
| MDA-MB-453 | 17 | 61 | 65 | 88 | 80 | 70 | 101 |
| MDA-MB-361 | 17 | 63 | 67 | 64 | 76 | 105 | 99 |
| BT474 | 25 | 27 | 29 | 60 | 21 | 78 | 91 |
| SK-BR-3 | 33 | 33 | 40 | 73 | 51 | 82 | 89 |
| SK-OV-3 | 17 | 77 | 85 | 87 | 91 | 97 | 99 |

[a] Based on FACS assays using MAb 4D5 and fluorescence-labeled goat antimurine IgG1 polyclonal Ab.
[b] Based on crystal violet staining of cell monolayers after 5 days exposure to 10 μg/ml of the indicated MAb.
Values are expressed as a percentage of the results obtained with untreated control cultures.
Estimated standard error ≤10%.

growth inhibition (Table 2). Based on these data, muMAb 4D5 was chosen as the most promising MAb for further characterization and possible use as an anticancer agent.

The antiproliferative properties of muMAb 4D5 were further explored by using it in conjunction with other antitumor agents against cultured tumor cell lines. As discussed previously, expression of p185HER2 appears to correlate with TNF-α resistance; thus, it was of interest to assess the use of anti-p185HER2 MAb along with TNF-α against cultured cells [39]. When treated with both muMAb 4D5 and TNF-α, two breast cancer cell lines that express high levels of p185HER2 showed significantly more growth inhibition (in fact, cytotoxicity was observed) in comparison with the growth inhibition seen with either agent alone (Figure 2). With tumor cell lines that express low levels of HER2, muMAb 4D5 caused no significant growth inhibition, consistent with earlier results; and the combination of muMAb 4D5 and TNF-α was no more effective than TNF-α alone.

The effect of muMAb 4D5 treatment in combination with the cytotoxic chemotherapeutic agent cis-platinum (CDDP) was also evaluated in the in vitro cell growth inhibition assay. In these studies [summarized in 40], muMAb 4D5 significantly enhanced the toxicity of CDDP to SK-BR-3 cells. Indeed, the CDDP dose associated with 50% cell viability was approximately 0.5 μg/ml with CDDP alone, but was 0.05 μg/ml when combined with muMAb 4D5. In contrast, muMAb 6E9, which as previously noted does not cause growth inhibition, did not add to the cytotoxic effect of CDDP.

### *3.3. Antiproliferative effects on tumor xenografts*

In addition to its inhibitory activity on cultured tumor cells in vitro, muMAb 4D5 was able to inhibit xenografted human tumor growth in an in vivo mouse model system. In this model, grafts of approximately 1 mg of the Murray human breast tumor, which expresses high levels of p185HER2, were implanted into the subrenal capsule of nude mice. After 7 days to allow for tumor take, the mice were given intravenous injections of muMAb 4D5 at a dose of 36 mg/kg. As controls, some mice received instead either PBS or an irrelevant isotype-matched murine monoclonal antibody, 5B6, which is directed against gp 120 from HIV, also at a dose of 36 mg/kg. Following this day 7 injection, the mice were given repeat injections on days 10 and 13, and then were sacrificed on days 20 or 34. Tumors were then existed and their sizes were obtained by gross measurement and weighing. The results of muMAb 4D5 treatment in this xenograft model are shown in Table 3. muMAb 4D5 caused significant growth inhibition of the xenografted human tumors at either the day 20 or day 34 endpoints; indeed, at day 20, the xenografts were four- to fivefold smaller with 4D5 treatment than with either the PBS or irrelevant MAb controls.

Just as muMAb 4D5 was found to greatly enhance the effect of CDDP in

inhibiting tumor cell growth in vitro (see above), it appears that muMAb 4D5 similarly enhances the toxicity of CDDP in this in vivo xenograft model as well (D. Slamon, personal communication).

In addition to confirming the antiproliferative effects of muMAb 4D5 in an in vivo setting, this xenograft model has been used in conjunction with $^{125}$I-labeled muMAb 4D5 to characterize the localization of muMAb 4D5 to tumors overexpressing HER2 (see Localization Studies).

Finally, $^{131}$I-labeled muMAb 4D5 immunoconjugates have also been constructed, and in a similar tumor graft model were found to localize specifically and rapidly to the engrafted tumors and to cause marked growth inhibition (K. De Santes, personal communication).

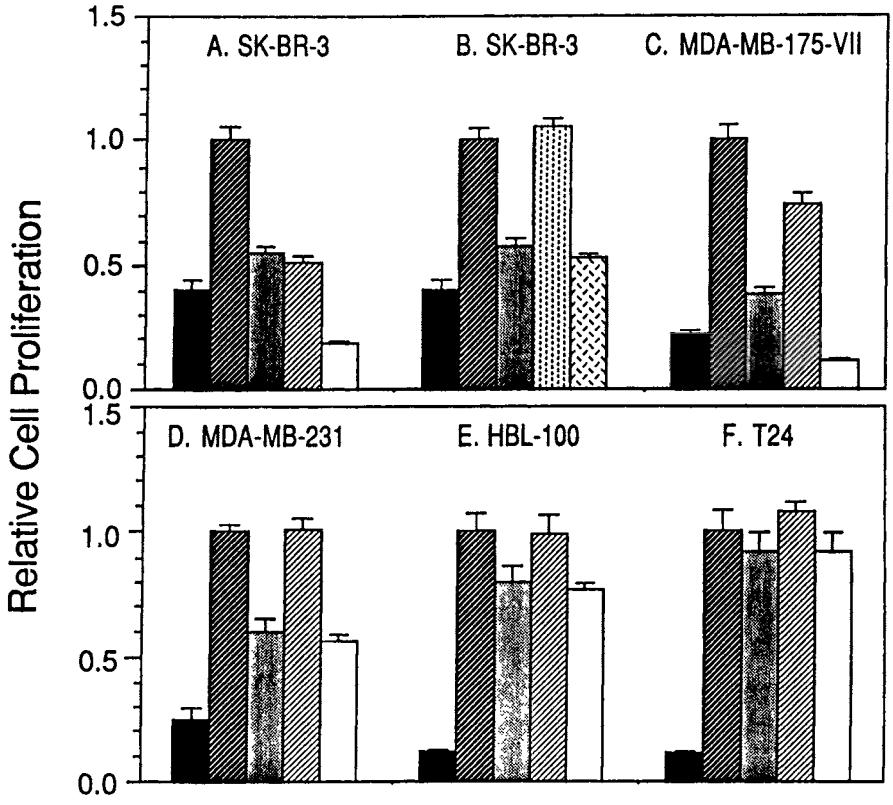


*Figure 2.* MAb 4D5 enhances TNF-α toxicity to breast cancer cell lines. Solid bars = cell number at initiation of assay; dark crosshatching = untreated control; dark stipples = TNF-α alone; light crosshatching = MAb 4D5; open bars = MAb 4D5 + TNF-α. B: Lack of growth inhibition of SK-BR-3 cells by MAb 40.1.H1 (anti-HBsAg; light stipples) and failure of MAb 40.1.H1 to enhance sensitivity to TNF-α (broken crosshatching). Breast cancer cell lines SK-BR-3 and MDA-MB-175-VII overexpress p185HER2 (see Table 2). Breast cancer cell lines MDA-MB-231 and HBL-100 and bladder cancer cell line T24 express low levels of p185HER2. The assays were performed as described in Hudziak et al. [39].

*Table 3.* MuMAb 4D5 inhibits the growth of a human breast tumor (murray) in athymic mice[a]

| | Tumor weight (mg)[b] | |
|---|---|---|
| Group (n = 4) | Day 20 | Day 34 |
| PBS | 6.79 ± 9.79 | 36.0 ± 30.7 |
| Control IgG (muMAb5B6) | 7.11 ± 5.48 | 88.1 ± 91.4 |
| muMAb4D5 | 1.48 ± 1.10 | 6.5 ± 6.4 |

[a] Administered as equally divided intravenous doses on days 7, 10, and 13 post tumor implantation.
[b] Data are mean ± standard deviation (SD) (n = 4).

### *3.4. How does muMAb 4D5 inhibit tumor proliferation?*

While it is clear that muMAb 4D5 can inhibit the growth of tumor cell lines in vitro and of xenografted tumors in vivo, it is less clear by what mechanisms(s) muMAb 4D5 acts. Again, the results from treating cultured cell lines with muMAb 4D5 (and the other anti-p185HER2 MAbs) indicate that the growth inhibition effect largely correlates with the degree of p185HER2 expression. It is reasonable to postulate that muMAb 4D5 interferes with the growth-promoting signal mediated by high levels of p185HER2. This might occur if muMAb 4D5 inhibited binding of an endogenous growth factor ligand, either secreted by the tumor cells themselves in an autocrine fashion or present in cell culture media. Another possibility is that muMAb 4D5 inhibits the ligand-independent, constitutive tyrosine kinase activity of overexpressed p185HER2; for example, muMAb 4D5 binding might directly alter specificity or activity of the tyrosine kinase, or might induce downmodulation of p185HER2. Still other mechanisms can be imagined, involving various types of interactions between antibody and receptor, or among antibody, ligand, and receptor.

Although these possibilities cannot be distinguished at present, there is evidence to suggest that muMAb 4D5 binding does trigger a series of events similar to that expected for a specific ligand. MuMAb 4D5 binding was found to induce downmodulation of cell surface p185HER2 receptor levels by twofold in the high p185HER2-expressing SK-BR-3 cells [41]. Concomitant with receptor downmodulation, these cells displayed rapid internalization and intracellular accumulation of muMAb 4D5 in an intact state. In contrast, the binding of muMAb 6E9, which lacks antiproliferative activity (see above), did not cause p185HER2 downmodulation in SK-BR-3 cells, nor was any significant muMAb 6E9 internalization or intracellular accumulation observed. In addition, muMAb 4D5 binding increases the tyrosine phosphorylation of p185HER2, presumably via autophosphorylation; p185HER2 phosphorylation was not stimulated by muMAb 6E9. These findings are consistent with the view that muMAb 4D5 behaves much like a ligand for p185HER2 in that it is internalized, causes receptor down-

modulation, and to some extent activates the tyrosine kinase domain. Since muMAb 4D5 inhibits rather than stimulates tumor cell growth, muMAb 4D5 may be a partial agonist or mixed agonist/antagonist of the p185HER2 receptor: Its ability to mimick ligand activation is incomplete and/or it competes with the actions of a more potent physiologic ligand.

### *3.5. Other anti-p185HER2 monoclonal antibodies*

Thus far we have discussed our efforts at developing and characterizing monoclonal antibodies directed against p185HER2, with particular focus on the interesting properties of muMAb 4D5. Other groups have also reported their work with monoclonal antibodies directed against of p185HER2 or rat p185*neu* [36,42–47]. MAb 7.16.4 inhibits NIH3T3 cells transformed with the activated rat *neu* gene in soft agar growth assays and also inhibits their growth in nude mice; this MAb also causes p185HER2 downmodulation [42,43]. Similarly, MAbs 3D2 and 9G6 also inhibit soft agar colony growth in *neu*-transformed NIH3T3 cells and induces p185HER2 downmodulation [45]. It is not yet clear whether any of these MAbs also stimulate receptor tyrosine kinase activity. On the other hand, MAbs B10 and H4 do induce tyrosine phosphorylation of p185HER2, as well as rapid downmodulation [46]. MAb 94 also displays ligand-like activity on NIH3T3 cells expressing a chimeric receptor containing extracellular and transmembrane domains derived from p185HER2 and a tyrosine kinase domain from EGFR [36]; binding by MAb 94 to the extracellular domain of p185HER2 stimulates the chimera's EGFR tyrosine kinase. The effect of these agonistic MAbs B10, H4, and 94 on tumor cell growth was not reported. Recently, a MAb with properties similar to 4D5 was described [47]. MAb MGR3 internalizes, stimulates p185HER2 phosphorylation, and inhibits the in vitro growth of a p185HER2-overexpressing lung adenocarcinoma cell line.

## 4. Humanized anti-p185HER2 monoclonal antibodies

### *4.1. Rationale*

As discussed above, the anti-p185HER2 muMAb 4D5 has shown significant antiproliferative effects on tumor cells in vitro and in an in vivo xenograft model, indicating its potential as an anticancer agent. However, it can be imagined that a monoclonal antibody with specificity and binding characteristics equivalent to muMAb 4D5, but of human rather than mouse derivation, might prove still more effective against human malignant diseases. One problem with mouse antibodies is immunogenicity: Clinical experience with murine monoclonal antibodies thus far suggests that a human antimouse antibody (HAMA) or antiglobulin response frequently occurs following the administration of mouse antibodies and can compro-

mise therapeutic efficacy [for review see 48]. The antimouse antibodies often alter the clearance and distribution of the therapeutic MAb; additionally, they may neutralize the therapeutic MAb, interfere with its binding, or enhance toxicity via immune complex formation. Another possible problem with mouse antibodies is lack of immunoreactivity: Most murine MAbs do not activate human effector mechanisms, such as complement-dependent cytotoxicity and antibody-dependent cell-mediated cytotoxicity (ADCC). This inability to induce cytoxicity against tumor cells bearing the target antigen has doomed many attempts at cancer therapy using murine MAbs. In contrast, of course, the efficacy of muMAb 4D5 may not require inducing host effector mechanisms, since muMAb 4D5 directly inhibits tumor growth via its interaction with the p185HER2 oncoprotein. Nevertheless, a monoclonal antibody capable of disrupting tumor cell function while simultaneously triggering an immune response to the tumor might prove still more effective in the treatment of cancer.

For these reasons, 'humanized' versions of MAb 4D5 were sought, using genetic engineering techniques to replace murine immunoglobulin sequences with human sequences while retaining those sequences critical to antigen binding.

### *4.2. Construction of humanized versions of MAb 4D5*

A set of 'humanized' versions of the murine MAb 4D5 were constructed by combining the antigen-binding sequences of muMAb 4D5 with human variable-region framework sequences along with human IgG1 constant region domains [49]. The IgG1 isotype was selected to maximize complement-dependent cytotoxicity and ADCC. To do this, the variable-region coding sequences ($V_H$ and $V_L$) of muMAb 4D5 were cloned from the corresponding hybridoma and sequenced. The $V_H$ and $V_L$ sequences were then used to design a humanized version, huMAb 4D5-1. For this molecule, the antigen-binding loops (complementarity determining regions or CDRs) of the murine antibody were retained, while consensus human sequences were substituted for the framework regions comprising the remainder of the variable region. The construction of this humanized molecule was carried out by a gene-conversion mutagenesis strategy. Briefly, long preassembled synthetic oligonucleotides representing the desired humanized sequences were annealed to a single-stranded DNA template containing the muMAb 4D5 $V_H$ and $V_L$ coding sequences; heteroduplex DNA was then synthesized in vitro and transfected into *E. coli*; finally, clones containing humanized $V_H$ and $V_L$ were isolated and their sequences were verified. The resulting humanized antibody-coding constructs were then transiently expressed in the human fetal kidney 293 cell line.

In addition to the most humanized version, huMAb 4D5-1, in which only the CDRs derive from muMAb 4D5, a set of seven additional humanized variants were constructed. In these variants, selected human residues in the

framework regions were replaced by residues from muMAb 4D5. This was done because framework residues can make significant contributions to antigen binding, either by direct contact with antigen or by influencing CDR conformation. Molecular modeling was used to identify appropriate residues for substitution. Five $V_H$ residues and two $V_L$ residues were selected in this way, and these were converted back to the muMAb 4D5 sequence by site-directed mutagenesis.

A total of eight humanized variants, designated huMAb 4D5-1 to 8, were ultimately constructed. Table 4 lists the amino-acid sequence differences among the antibodies. Binding affinity with the extracellular domain of p185HER2 is also shown. Of note, the best binding was achieved by huMAb 4D5-8, which is the most similar to muMAb 4D5; indeed, huMAb 4D5-8 showed threefold higher affinity than muMAb 4D5 itself. In contrast, the poorest binding was seen with the most humanized variant, huMAb 4D5-1, which contains muMAb 4D5 residues in the CDRs only. This confirms the importance of selected framework sequences in antigen binding. Table 4 also gives the antiproliferative activity of the humanized variants, using the same assay with the p185HER2-overexpressing breast cancer cell line SK-BR-3 that was discussed earlier in this chapter. It can be seen that most of the humanized variants retained significant antiproliferative activity. Additionally, binding affinity was found not to be the sole determinant of the antiproliferative effect.

To determine the effectiveness of humanized anti-p185HER2 antibodies in activating ADCC, huMAb 4D5-8 was compared with muMAb 4D5. The

*Table 4.* $p185^{HER2}$ ECD binding affinity and antiproliferative activities of MAb4D5 variants

| | $V_H$ residue[a] | | | | | $V_L$ residue[a] | | | |
|---|---|---|---|---|---|---|---|---|---|
| MAb4D5 Variant | 71 FR3 | 73 FR3 | 78 FR3 | 93 FR3 | 102 CDR3 | 55 CDR2 | 66 FR3 | $K_d$[b] nM | Relative cell proliferation[c] |
| huMAb4D5-1 | R | D | L | A | V | E | G | 25 | 102 |
| huMAb4D5-2 | Ala | D | L | A | V | E | G | 4.7 | 101 |
| huMAb4D5-3 | Ala | Thr | Ala | Ser | V | E | G | 4.4 | 66 |
| huMAb4D5-4 | Ala | Thr | L | Ser | V | E | Arg | 0.82 | 56 |
| huMAb4D5-5 | Ala | Thr | Ala | Ser | V | E | Arg | 1.1 | 48 |
| huMAb4D5-6 | Ala | Thr | Ala | Ser | V | Tyr | Arg | 0.22 | 51 |
| huMAb4D5-7 | Ala | Thr | Ala | Ser | Tyr | E | Arg | 0.62 | 53 |
| huMAb4D5-8 | Ala | Thr | Ala | Ser | Tyr | Tyr | Arg | 0.10 | 54 |
| muMAb4D5 | Ala | Thr | Ala | Ser | Tyr | Tyr | Arg | 0.30 | 37 |

[a] Human and murine residues are shown in one-letter and three-letter amino acid code, respectively.

[b] $K_d$ values for the $p185^{HER2}$ ECD were determined using the method of Friguet et al. [50] and the standard error of each estimate is $\leq \pm 10\%$.

[c] Proliferation of SK-BR-3 cells inclubated for 96 hours with MAb4D5 variants shown as a percentage of the untreated control as described [7]. Data represent the maximal antiproliferative effect for each variant (see Figure 3) calculated as the mean of triplicate determinations at a MAb4D5 concentration of 8 μg/ml. Data are all taken from the same experiment and the estimated standard error is $\leq \pm 15\%$.

*Table 5.* Selectivity of antibody-dependent tumor cell cytotoxicity mediated by huMAb4D5-8

| Effector target ratio[b] | WI-38[a] | | SK-BR-3 | |
|---|---|---|---|---|
| | muMA4D5 | huMAb4D5-8 | muMAb4D5 | huMAb4D5-8 |
| A.[c] 25:1 | <1.0 | 9.3 | 7.5 | 40.6 |
| 12.5:1 | <1.0 | 11.1 | 4.7 | 36.8 |
| 6.25:1 | <1.0 | 8.9 | 0.9 | 35.2 |
| 3.13:1 | <1.0 | 8.5 | 4.6 | 19.6 |
| B. 25:1 | <1.0 | 3.1 | 6.1 | 33.4 |
| 12.5:1 | <1.0 | 1.7 | 5.5 | 26.2 |
| 6.25:1 | 1.3 | 2.2 | 2.0 | 21.0 |
| 3.13:1 | <1.0 | 0.8 | 2.4 | 13.4 |

[a] The sensitivity to ADCC of two human cell lines (WI-38, normal lung epithelium; and SK-BR-3, human breast tumor cell line) are compared. WI-38 expresses a low level of $p185^{HER2}$ (0.6 pg $p185^{HER2}$/μg cell protein) and SK-BR-3 expresses a high level of $p185^{HER2}$ (64 pg $p185^{HER2}$/μg cell protein), as determined by ELISA [44].

[b] ADCC assays were carried out as described [15]. Effector to target ratios were of IL-2-activated human peripheral blood lymphocytes to either WI-38 fibroblasts or SK-BR-3 tumor cells in 96-well microtiter plates for 4 hours at 37°C. Values given represent percent specific cell lysis as determined by $^{51}Cr$ relases. Estimated standard error in these quadruplicate determinations was $\leq \pm 10\%$.

[c] Monoclonal antibody concentrations used were 1 μg/ml (A) and 0.1 μg/ml (B).

MAbs were added to either SK-BR-3 cells or to low p185HER2- expressing WI-38 cells, along with a range of activated T-lymphocyte effector cells (Table 5). As expected, the humanized antibody was much more effective (at least fivefold) in mediating ADCC against SK-BR-3 cells than murine 4D5, while mediating little ADCC against WI-38 cells.

## 5. Towards anti-p185HER2 immunotherapy with MAB 4D5

### *5.1. Localization studies*

In order to assess quantitatively the localization of murine MAb 4D5 to sites of tumor in vivo, the biodistribution in mice of $^{125}I$-labeled muMAb 4D5 was evaluated following intravenous (IV) and intraperitoneal (IP) administration. Eighteen nude mice received 1-mg xenografts of the p185HER2-overexpressing Murray human breast tumor, implanted under the subrenal capsule and allowed to vascularize and develop for 40 days. $^{125}I$-labeled muMAb 4D5 was administered at a dose of 30 μg/kg (about 35 μCi/mouse) by IV (n = 9) or IP (n = 5) bolus. $^{125}I$-labeled murine monoclonal antibody 5B6, an irrelevant control antibody, was administered at a similar dose by IV bolus (n = 4). Mice receiving radiolabeled muMAb 4D5 were sacrificed at 5 minutes, 3 hours, 24 hours, 3 days, and 7 days following administration of the anbibody. Mice receiving the control antibody 5B6 were sacrificed at 24 hours and at 7 days. Blood samples collected immediatedly prior to

sacrifice were assayed for total radioactivity, and plasma samples were assayed for TCA-precipitable radioactivity. After sacrifice, the mice were frozen, embedded in 4% carboxymethylcellulose, and cut along the sagittal plane into whole-body sections of 20-μ thickness. These sections were then used for autoradiography.

The vast majority of radioactivity measured in plasma samples was TCA precipitable. The accumulation of $^{125}$I-labeled muMAb 4D5 in various body tissues and fluids following IV administration is given in Table 6. Similar results were obtained following IP administration. With either route of administration, the autoradiographs confirmed that muMAb 4D5 localization to the tumor was low after 5 minutes but then progressively increased over the initial few days. A heterogeneous distribution of muMAb 4D5 within the tumor was observed during the first 24 hours, but became increasingly uniform at days 3 and 7. In contrast, no uptake of the control antibody was evident in tumor tissue. These findings demonstrate that muMAb 4D5, given either IV or IP, rapidly and specifically localizes to p185HER2-overexpressing tumor xenografts in vivo, with relatively little antibody accumulation in other body compartments.

### *5.2. Plans for clinical trials*

The studies described in this chapter indicate that the anti-p185HER2 muMAb 4D5 is a promising antiproliferative agent that may be useful in the treatment of human cancers involving p185HER2 overexpression. Therefore, we are initiating clinical trials with muMAb 4D5. The first studies will be Phase I single- and multiple-dose trials in which the antibody is administered intravenously to breast cancer patients and intraperitoneally to ovarian cancer patients. Patients considered for this trial will have their biopsy or other pathologic materials assayed for level of p185HER2 expression by immunohistochemistry. Only those patients with p185HER2 levels of 3+ (on a 0–4+ scale), which usually corresponds to >5 copies of the HER2 protooncogene, will be enrolled. These Phase I studies will be used to evaluate acute and chronic toxicities, the degree of HAMA response, the extent of tumor localization, the pharmacokinetic parameters, and the optimal drug dosing schedule.

If muMAb 4D5 is well tolerated and good tumor localization is observed, a number of additional treatment strategies will be considered. For example, rather than single-agent use, muMAb 4D5 may be tried in combination with established cytotoxic chemotherapeutic drugs. Another approach being developed is the construction of radioimmunoconjugates, using muMAb 4D5 linked to a toxic radionuclide such as $^{131}$I, $^{90}$Y, or $^{186}$Re. This will combine the growth-inhibiting properties of muMAb 4D5 with radioimmunotherapy. In addition, since the immunogenicity of murine antibodies is likely to preclude the chronic administration of muMAb 4D5, we intend to conduct trials with one of the humanized versions of MAb 4D5 discussed

*Table 6.* $^{125}$-Ilabeled murine MAb 4D5 accumulation in a p185HER2-overexpressing human breast tumor in nude mice

| Tissue/fluid | 5 min | 3 hr | 24 hr | 3 days |
|---|---|---|---|---|
| Ventricular blood | 28,050 | 19,865 | 9,778 | 2,068 |
| Liver | 21,472 | 6,882 | 2,928 | 1,249 |
| Kidney | 8,946 | 4,289 | 2,354 | 1,086 |
| Tumor | <265 | 3,048 | 13,173 | 2,494 |
| Tumor/blood ratio | ~0 | 0.2 | 1.4 | 1.2 |
| Tumor/kidney ratio | ~0 | 0.7 | 5.8 | 2.3 |

[a] Disintegrations/min/mg tissue or fluid (dpm/mp) following IV administration of 35 mCi of $^{125}$I-labeled muMAa 4D5.

earlier; this will potentially allow repeated dosing and possibly improve therapeutic efficacy by the mechanisms previously discussed. The clinical plan for humanized MAb 4D5 will likely be similar to that described for the murine antibody. Finally, since p185HER2 overexpression has been observed in other neoplasms, similar trials in non-small-cell lung cancer, gastric cancer, endometrial cancer, and other malignant diseases are anticipated.

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# 11. A chimeric EGFR/*neu* receptor in studies of *neu* function

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## 1. Structure and expression of the chimeric EGFR/*neu* receptor

Soon after the characterization of the *neu* oncogene by Weinberg and colleagues [1,2], the discovery of its amplification in breast cancer by Slamon and others [3–6] created an intense interest in its cellular function. Defining the role of *neu* in cellular transformation became a high-priority task in many laboratories around the world.

The approach used in our studies was to produce a recombinant receptor that would enable ligand regulation of the *neu* tyrosine kinase. The hypothesis that receptor tyrosine kinases (RTK) are composed of functional domains that act as modular assemblies had already been supported by experimental data [7,8]. We fused the extracellular, ligand-binding portion of the EGFR to intracellular domains of the *neu* tyrosine kinase with the prediction that EGF would regulate the tyrosine kinase activity of the recombinant receptor and induce signal transduction typical for activated *neu*.

The chimeric EGFR/*neu* construct was made utilizing a conserved restriction site in the juxtamembrane domain adjacent to the transmembrane domain of the EGFR and *neu* cDNAs [9]. This region is highly conserved between the EGFR and *neu* proteins. The resulting expression vector, pSV2EGFR/*neu* (Figure 1A), was used to transfect NIH 3T3 mouse fibroblasts. With the help of *neu*-specific antibodies and [$^{125}$I]-EGF binding, a number of cell clones expressing the EGFR/*neu* hybrid protein were isolated (Figure 1B). Radioactive EGF binding analysis indicated that cells expressing the hybrid protein have specific high-affinity receptors for EGF on their surface. Based on these experiments the approximate number of receptors per cell in characterized clones (termed *NEN cells* for NIH3T3 EGFR/*neu*), ranged from $4 - 20 \times 10^5$. This is well above the estimated number of EGFR in NIH 3T3 cells ($3 - 6 \times 10^3$). As expected, the apparent molecular weight of the hybrid protein was slightly higher than that of *neu* protein, approximately 190,000 (Figure 2). By immunofluorescence we showed that the protein is located on the plasma membrane in a correct orientation. In all subsequent experiments where NIH3T3 or its neomycin-

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

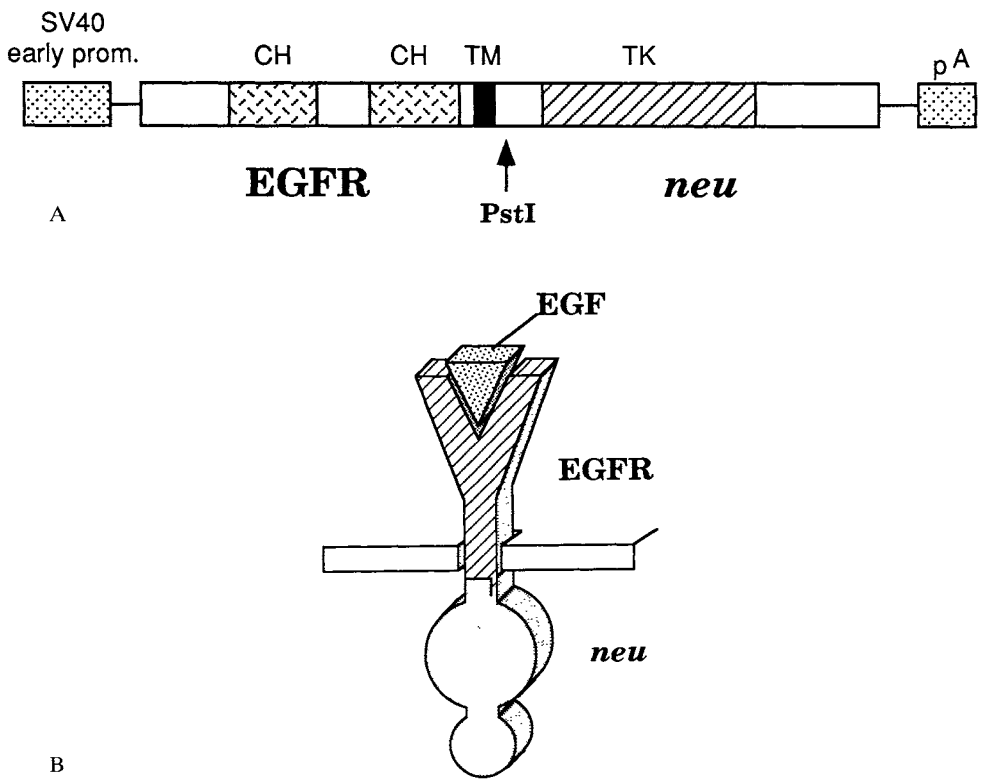


*Figure 1.* A: Schematic drawing of the expression vector pSV2EGFR/*neu* used to transfect NIH 3T3 mouse fibroblasts. The conserved *PstI* site used to cut and ligate the EGFR and *neu* component cDNA sequences is marked. CH = cysteine-rich domain; TM = transmembrane domain; TK = tyrosine kinase domain; pA = polyadenylation site. B: Structure of the EGFR/*neu* chimeric receptor. EGFR extracellular, transmembrane, and protein kinase C domains are fused to the intracellular tyrosine kinase and carboxyl terminal domains of rat *neu* protein.

resistant derivative NN (for NIH neo) cells were used as controls, their EGF-dependent responses were found to be absent or barely detectable when compared to the receptor-transfected cells.

## 2. Receptor characteristics and autophosphorylation

As is well established for EGFR, EGFR/*neu* also formed two classes of receptors with different affinities for EGF (Figure 3). In cells expressing high amounts of both *neu* and EGF receptors, heterodimers with an approximately a 100-fold higher affinity to EGF than the normal high-affinity state of the EGFR have been reported [10]. No evidence of such extremely

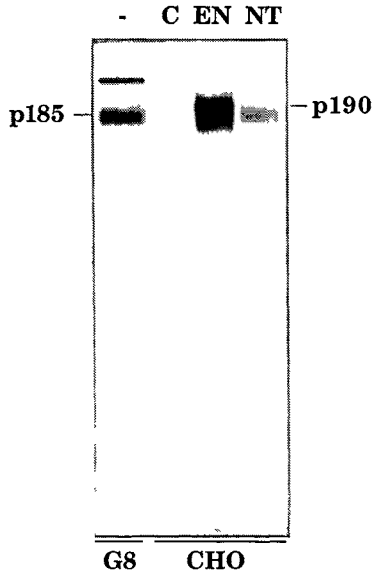


*Figure 2.* SDS-PAGE analysis of anti-*neu* immunoprecipitates from CHO cells and G8 cells. G8 cells expressing an amplified *neu* protooncogene [23], C cells expressing only the neomycin-resistance marker gene, EN cells expressing the chimeric EGFR/*neu* hybrid protein, and NT cells expressing the *neu* oncogene were labeled with [$^{35}$S]-methionine, lysed, and immunoprecipitated with the anti-*neu* antiserum.

high-affinity binding was obtained in experiments with the NEN cells, apparently due to minimal EGFR expression in the cells. The formation of heterodimers has also been suggested to be responsible for *neu* transphosphorylation by ligand-activated EGFR [11–13]. Wast *neu* overexpression may also lead to cell transformation in the absence of added ligand [10,14–16].

Ligand activation of the EGFR/*neu* receptor tyrosine kinase in the transfected cells was monitored using antiphosphotyrosine antibodies, which specifically recognize tyrosyl side chains with a covalently attached phosphate group. With this method, it was shown that autophosphorylation of the hybrid protein occurs only in the presence of EGF or TGF-α (Figure 4) [9]. However, the most highly expressing cell clone, NEN7, was shown to have residual tyrosine kinase activity also in the absence of ligand. This suggested that there might be an upper limit for receptor number above which the normal regulatory signals are altered. The importance of receptor number was studied by cloning the hybrid cDNA under the Moloney murine leukemia virus LTR promoter [17], which provides a higher constitutive rate of transcription than the SV40 early promoter. This construct was translated into cells and two resulting clones, LTREN1 and LTREN2, were analyzed in the same manner as the NEN clones. The approximate receptor numbers

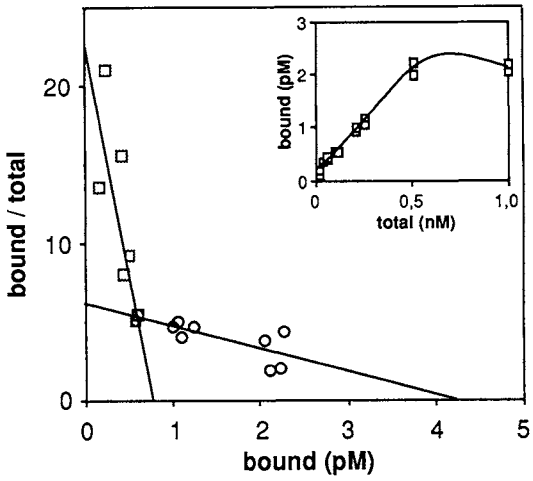


*Figure 3.* Scatchard plot of NEN37 cells. The [$^{125}$I]-EGF binding isotherms determined for NEN37 cells expressing chimeric EGFR/*neu* are presented by the method of Scatchard.

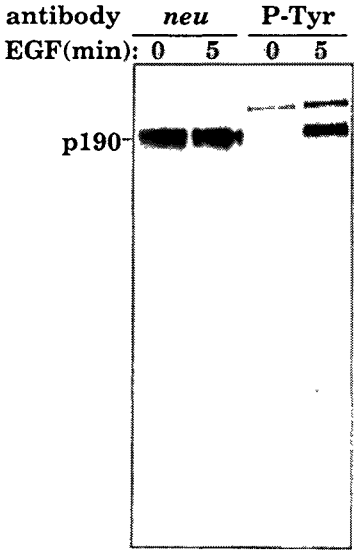


*Figure 4.* SDS-PAGE analysis of anti-*neu* and anti-PTyr immunoprecipitates from metabolically labeled NEN49 cells that express chimeric EGFR/*neu* receptors. [$^{35}$S]-methionine labeled cells were lysed and immunoprecipitated with the anti-*neu* antiserum or with the anti-phosphotyrosine antibodies (P-tyr), as shown.

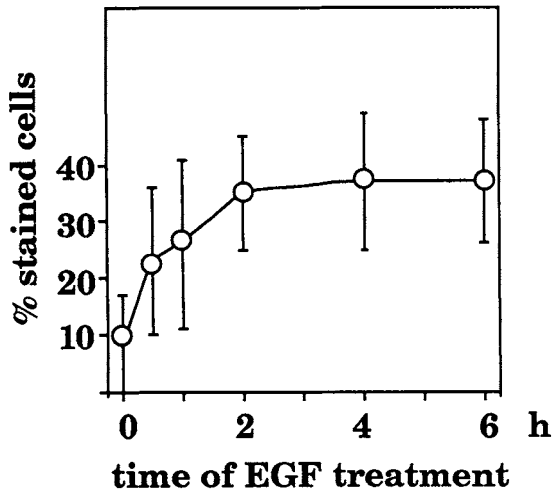


*Figure 5.* Stimulation of DNA synthesis and its dependence on the length of EGF treatment. Cells were seeded at $10^5$ cells/small plate. After 24 hours incubation in medium containing 1% FCS, the cells were stimulated by the addition of 10 nM EGF for the indicated times, the growth factor was washed off, and DNA synthesis was analyzed 20 hours later using 1.5-hour pulses with BrdU and staining with anti-BrdU antibodies.

in cells of these clones were of the same magnitude as in NEN7 cells, about $\sim 6 - 15 \times 10^5$ per cell, yet no autophosphorylation was seen in the absence of ligand. Antiphosphotyrosine immunoblotting gave similar results [18]. Thus it may be concluded that within these conditions the *neu* tyrosine kinase in general retained its dependence on ligand for activation.

### 3. Growth regulation

In all assays tested, the EGFR/*neu*-expressing cells were strongly influenced by EGF. Cellular morphology changed from flat to highly elongated, and DNA synthesis of serum-starved cells increased after the addition of EGF (Figure 5). The responses were dependent on the concentration of EGF in the assay [19], and the threshold concentration of EGF for maximum induction was dependent on the number of EGFR/*neu* receptors [20]. Growth in soft agar was also dependent on EGF and was correlated with receptor number/cell. Furthermore, soft agar growth was similar when TGF-α was used instead of EGF [9].

Our studies indicated that in the presence of EGF, the EGFR/*neu* receptor gave two- to fivefold less soft agar colonies than the *neu*NT oncoprotein and about tenfold less than the c-Ha-*ras* oncogene [9,17]. In all these assays cells of clone NEN7 had higher basal activity than other EGFR/*neu*-expressing cell clones. This is most probably due to the higher basal

activity of its chimeric tyrosine kinase, a peculiarity of this individual cell clone and not a general property of the chimeric receptor.

By contrast, the *neu* protooncogene did not show kinase activity or transforming properties when expressed at similar levels in NIH 3T3 cells. Other studies have suggested that the normal *neu* protooncogene can transform cells if it is sufficiently overexpressed [16]. However, the level of expression obtained in these studies is apparently higher than in our experiments and in experiments where no transformation was seen, even when the amplification level of *neu* was increased with selection [21–23]. Parallel with our study [9], Lee et al. [24] reported a construct in which instead of *neu*, the c-*erb*B-2 cytoplasmic domain was used. In this vector, the EGFR/c-*erb*B-2 joining site was constructed using synthetic oligonucleotides introduced into the proximal portion of the extracellular domain. The results obtained with this construct were similar to ours in all relevant aspects.

These results suggested that the *neu* protooncogene possesses mitogenic and transforming properties in the presence of a ligand that stimulates its tyrosine kinase activity, and they provided the first model for studies of the function of the *neu* tyrosine kinase. These experiments also provide evidence that the growth-promoting properties of the *neu* tyrosine kinase, and therefore apparently of the intact *neu* protein, are similar to those of EGFR. Also, they confirm the hypothesis that heterologous domains of *neu* and EGFR can be combined to form a functional receptor. Numerous other chimeras between different members of the RTK family and related proteins have also been constructed, mostly confirming the compatibility of domains [24–33], with only one highly instructive exception [34].

## 4. Cellular responses to *neu* kinase

When the growth-promoting effects of the *neu* tyrosine kinase were found to be identical with those of EGFR, we went on to test other effects of the ligand-activated *neu* tyrosine kinase. These studies had a dual purpose: (1) to further analyze the extent of functional similarities between *neu* and EGFR protein and (2) to find potential differences in cellular responses to ligand binding to the two receptors. It should be noted that coexpression of these two related receptors has been reported in various cell types [12,35].

Several morphological effects of EGF addition were observed in cells expressing the EGFR/*neu* hybrid protein. Time-lapse videography recorded extensive membrane ruffling, increased pinocytosis, and extension of lamellar footpads at the cell periphery. Using immunofluorescence microscopy, the extension of footpads was related to partial reorganization of cellular actin stress fibers [19]. More delayed changes were acquisition of an elongated shape, unordered orientation of the cells, and formation of

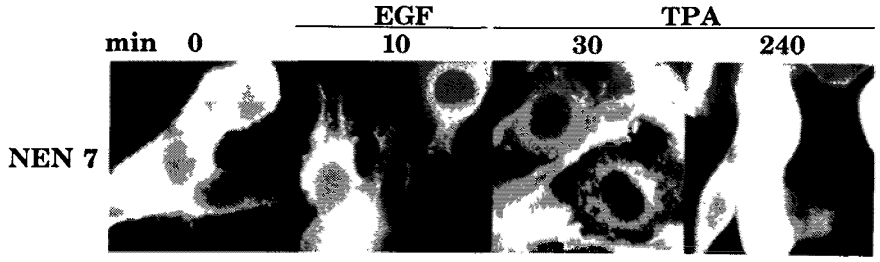


*Figure 6.* Immunofluorescence localization of receptor proteins after incubation with EGF or TPA. The NEN7 cells, which express the chimeric EGFR/*neu* receptor, were preincubated in medium containing 1% FCS and fixed immediately (0) or after further incubation in the presence of 50 nM EGF or TPA for the time periods shown. The receptors were localized using anti-*neu* antibody staining. After 10 minutes of EGF and 30 minutes of TPA incubation, the receptors were internalized into a punctate pattern of intracytoplasmic vesicles. At 240 minutes of TPA treatment, a major part of receptor fluorescence is again seen at the cell surface.

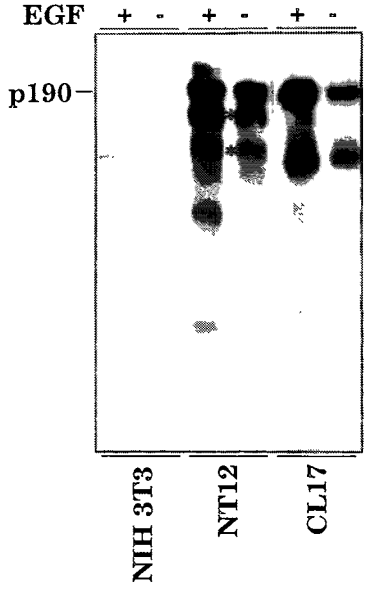


*Figure 7.* Comparison of tyrosine phosphorylation in starved and EGF-stimulated cells by Western blotting using anti-Ptyr antibodies. Serum-starved control NIH3T3 cells, NT12 cells expressing *neu* oncogene, and CL17 cells expressing EGFR were stimulated with 3 nM EGF for 30 minutes after a 15-minute, 300-μM sodium orthovanadate preincubation. Cultures were extracted in boiling electrophoresis buffer and 300 μg of cell lysate protein was used for analysis. After SDS-PAGE, tyrosyl phosphorylated proteins were identified by immunoblotting with anti-Ptyr antibodies [62] and [$^{125}$I] protein A. Tyrosine-phosphorylated proteins specific for the NT12 cells are marked by asterisks.

multilayered foci of cells [9,17,19,20]. Somewhat similar changes have been reported in mouse epithelial cells exposed to EGF [36].

The fate of the EGFR/*neu* receptor was monitored using antibodies and radioactive EGF binding. After EGF binding the EGFR/*neu* protein was seen to be rapidly internalized into a vesicular intracellular compartment and it underwent degradation (Figure 6). The half-life of the untreated EGFR/*neu* receptor was over 4 hours, and after EGF treatment it was about 50 minutes. Similar to the EGFR, TPA caused a transient internalization without a significant degradation of the chimeric receptor [19].

In collaboration with A. Pandiella and J. Meldolesi, we showed that other cellular proteins and pathways are involved in the *neu* tyrosine kinase-induced signal transduction. As is well established for the EGFR, the *neu* tyrosine kinase was also shown to induce phospholipid hydrolysis, an increase of intracellular calcium, and plasma membrane hyperpolarization [20]. These results show that upon ligand binding the chimeric EGFR/*neu* protein undergoes typical receptor downregulation and transduces cellular signals with characteristics similar to the EGF receptor [37–39].

Recently, we have analyzed *neu* tyrosine-kinase-expressing cells by phosphotyrosine immunoblotting. Tyrosine phosphorylated proteins were similar but not identical in epidermal growth factor (EGF)-stimulated cells expressing the human EGF receptor (EGFR) or a chimeric EGFR/*neu* receptor, but differed from phosphotyrosyl proteins constitutively expressed in *neu* oncogene-transformed cells (Figure 7). The *neu* oncoprotein in the latter cells was phosphorylated in tyrosine in a ligand-independent manner and had a shortened half-life in comparison with the normal *neu* protein. Several other phosphotyrosine-containing polypeptides were observed in cells expressing the recombinant receptors. Some of these may represent substrates for the *neu* tyrosine kinase, and their enhanced phosphorylation in the *neu* receptor-expressing cells in comparison with EGFR expressing cells may indicate differences in substrate specificities between the two receptors [18].

Interestingly, the molecular weights of the major phosphotyrosine-containing polypeptides of 145,000 and 124,000, specific for *neu* oncoprotein expressing NT12 cells, are very similar to the molecular weights of the recently discovered physiologically interesting substrates for the PDGFR and EGFR. These include phospholipase C$\gamma$ ($M_r$ 145,000) [40] and the GTPase activating protein (GAP, $M_r$ 124,000) [41–43]. Also, the $M_r$ 84,000 phosphotyrosyl polypeptide, which is detected in the *neu* oncoprotein-transformed cells and whose phosphorylation is increased in EGF-treated cells expressing the EGFR/*neu* receptor, migrates with the mobility of the phosphoinositol-3 kinase, which is a known substrate for the PDGFR [44]. The definitive identification of the polypeptides phosphorylated in our transfected cells will require further work using specific antibodies. The significance of various intracellular substrates and responses to ligand-induced activation of RTKs is presently under intensive investigation.

## 5. Regulation of receptor activity by activators of protein kinase C

Phorbol ester tumor promoters have been reported to block tyrosine-specific phosphorylation of the EGFR via activation of protein kinase C, which phosphorylates a target threonine residue (Thr 654) in the juxtamembrane region of the EGFR [45]. Phorbol ester treatment also leads to abolition of high-affinity binding sites of EGFR [46] and chimeric HER1-2 receptors [24]. In our experiments TPA pretreatment blocked the *neu*-specific kinase activity stimulated by EGF. However, tyrosyl-phosphorylated *neu* oncoprotein was about 50% decreased in TPA-treated cells, although earlier studies of Dobashi et al. [47] using immunoprecipitation of *neu* protein from [$^{32}$P]-labeled cells and immunocomplex kinase reactions showed that TPA affects only the normal *neu* protooncogene-encoded protein but not the oncoprotein. Our own results are likely to better represent the situation in intact cells, as the treated cells were directly lysed in denaturing conditions for analysis. Interestingly, in our experiments the phosphatase inhibitor okadaic acid did not affect phosphotyrosine in the *neu* oncoprotein. Yet, okadaic acid was as effective as TPA in preventing EGF-induced tyrosyl phosphorylation of the EGFR/*neu* receptor (Figure 8) [18]. Therefore the activities of the ligand-dependent *neu* tyrosine kinase and the *neu* oncoprotein tyrosine kinase are differentially inhibited by TPA and okadaic acid, which act by different routes [48,49].

## 6. Activation of cell growth-regulated genes

The effect of EGF in cells expressing the chimeric receptor was also seen as a rapid increase in the expression of growth-regulated genes, such as transcription factors *jun*B [19], c-*jun*, *fos*, glucose transporter, and ornithine decarboxylase [50]. Also, the activities of glucose transporter and ornithine decarboxylase enzymes were measured and found to be rapidly and strongly elevated [17,50]. In all these cases the response was reminiscent of that induced through the EGFR [51–53].

A larger selection of growth-regulated genes was tested for their induction by the *neu* tyrosine kinase. We used a library of 78 genes that are all rapidly activated in quiescent NIH 3T3 cells after serum addition [54]. Probing of filter-bound DNA from these plasmid clones with reverse-transcriptase-labeled cellular mRNA from various EGF-treated receptor-expressing and control cell lines showed that 28 of these genes responded to EGF in cell lines expressing similar amounts of EGFR/*neu* protein or EGFR. However, none of the genes was entirely specific to the signaling pathways used by the EGFR/*neu* receptor. Only qualitative differences in maximal induction were observed, and only in a total of four genes (Figure 9; ref. 55). Northern blotting and hybridization analysis performed for several of these mRNAs confirmed the dot-blot results. The significance of

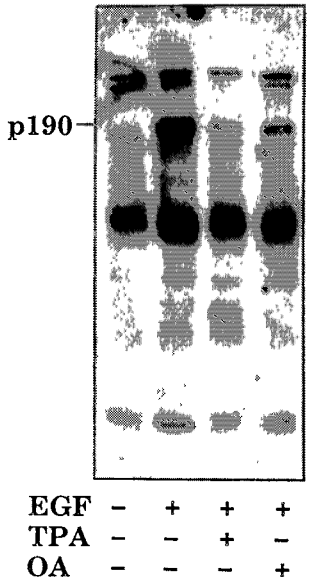


*Figure 8.* Effect of TPA and okadaic acid on the tyrosine phosphorylation of the EGFR/*neu* receptor. NEN37 cells expressing EGFR/*neu* receptors were serum-starved for 24 hours before treatment with 100 nM TPA or 1 μM okadaic acid (OCA). EGF was added to the cultures 30 minutes after the addition of TPA or okadaic acid and the cells were lysed 5 minutes later. Immunodetection of phosphotyrosine-containing proteins was performed as in Figure 7.

the few observed differences between the responses to EGFR/*neu* and EGFR receptors is unclear, especially when the biological function of the genes for which differences were seen in still largely unknown. In conclusion, then, these results emphasize the similarity of *neu* and EGFR receptors.

### 7. TPA inhibition of induction of immediate early genes by ligand-activated *neu*

As already mentioned, TPA inhibited receptor tyrosyl phosphorylation upon EGF binding. Long-term TPA treatment, known to downregulate protein kinase C [56], also prevented the induction of several rapidly activated mRNAs in the EGF-treated cells. Hybridization of reverse-transcriptase-labeled cellular mRNA to cDNAs representing serum-inducible genes showed that EGF stimulation of serum-starved NEN37 cells expressing EGFR/*neu* chimeric receptors resulted in a sequential activation of various mRNAs, including the *jun*B and N10 mRNAs. In the cells that had been exposed to TPA, most of these mRNAs no longer responded in a detectable manner [18].

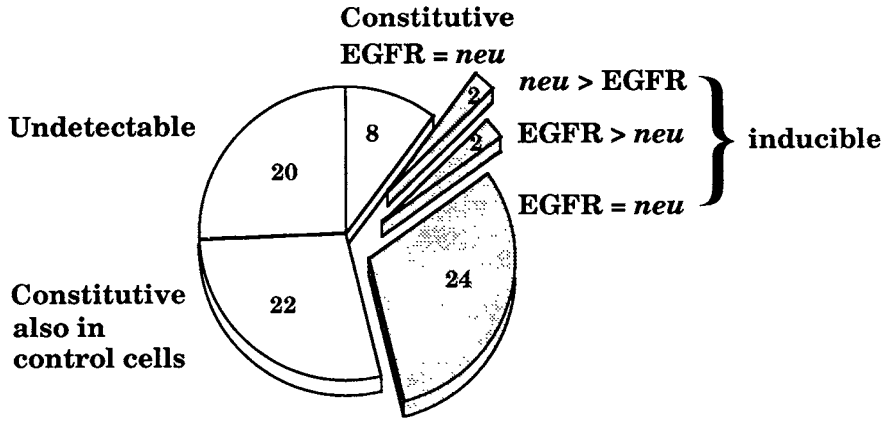


*Figure 9.* EGF activation of growth-regulated genes through signals mediated by the EGFR and EGFR/*neu* receptor. A panel of growth factor inducible genes was tested for their induction by the *neu* tyrosine kinase. Poly(A)+ RNA from quiescent cells stimulated with EGF was labeled into radioactive complementary DNA and hybridized to dot filters containing cDNA clones of 78 serum-inducible genes [54]. Several mRNAs were not detectably induced by EGF (undetectable; 20 genes), while some were constitutively expressed at a low level in these cells (8 genes) and also in control cells (22 genes). A total of 28 cDNAs were induced by EGF (grey sectors). None of the genes was entirely specific to the signaling pathways used by either of the two receptors. In maximal induction only four genes showed qualitative differences.

## 8. Expression of immediate early genes in normal and *neu*-transformed cells

Cells expressing the active *neu* oncogene generally had a low level of transcription of the immediate early genes [55]. Also, introduction of the active *neu* oncogene into cells already overexpressing the EGFR/*neu* receptor was able to inhibit 75–90% of the induction obtained by treatment with EGF as well as the stimulation obtained with platelet-derived growth factor and fetal calf serum [18,57]. This suggests that one aspect of malignant cell transformation may be the insensitivity of immediate early mRNA responses to growth factors. It should also be noted that some of the immediate early genes were constitutively expressed in the *neu*-transformed cells [57].

We studied the biosynthesis of the proteins encoded by genes whose expression was significantly increased by the ligand activation of *neu* tyrosine kinase and was blocked by the *neu* oncoprotein. The expression of the *jun* and *fos* oncogene-encoded polypeptides was analyzed by immunoprecipitation of serum-starved and EGF-stimulated cells expressing the *neu* oncogene. As expected on the basis of the results of mRNA analysis of the *neu* oncogene-transformed cells, the biosynthesis of the c-*jun* protein was somewhat elevated in the *neu* oncogene-expressing cells [50]. EGF treatment enhanced only slightly the synthesis of the *jun* and *fos* proteins in NIH

3T3 and NT11 cells, whereas the synthesis of these proteins was highly increased upon EGF treatment of the NEN37 cells [18].

The *jun* and *fos* proteins are components of the activator protein-1 (AP-1) complex, which binds to TRE element present in promoter regions of various TPA-inducible genes [58–60]. Interestingly, many of these TPA-inducible genes are also activated by the c-Ha-*ras* oncogene, apparently through the TRE element [61]. Our own studies have indicated that the *neu*-transformed cells also have an increased AP-1 activity [50].

## 9. Cells with activated *neu* show downregulation of PDGF receptors

Although comparisons of gene expression between mitogen-stimulated normal cells and transformed cells must be interpreted with caution, our experiments suggest that the downregulation of the immediate early mRNA response in the *neu* oncogene-expressing cells concerns both the PDGF receptor tyrosine-kinase-mediated signals and the more generalized activation signals obtained by serum stimulation. This downregulated response may be partly explained by a lack of the growth factor receptors from the cell surface, as shown by [$^{125}$I]-PDGF-AA and -BB binding assays [63]. These results were corroborated by metabolic labeling and immunoprecipitation of the PDGF β-receptors. PDGF α- and β-receptor mRNAs were decreased in the *neu* oncogene transformed cells in comparison with control cells expressing the *neu* protooncogene. Downregulation of the PDGF receptors and their mRNAs was also observed after EGF treatment of cells expressing a chimeric EGFR/*neu* receptor, where the *neu* tyrosine kinase is activated by ligand binding. These results showed that the *neu* tyrosine kinase can downmodulate PDGF receptor expression, and the effect is mediated via decreased mRNA levels.

## 10. Summary and future perspectives

As the factor binding to the *neu* protein has been unknown, it has not been possible to confirm experimentally the proposed growth-factor receptor-like functions of the *neu* protein. To approach this problem we constructed a recombinant receptor that would enable ligand regulation of the *neu* tyrosine kinase. The hybrid receptor consisted of the extracellular ligand binding, transmembrane and protein kinase C-substrate domains joined to the intracellular tyrosine kinase and carboxyl-terminal domains of the *neu* protein.

Several properties of NIH3T3 cells carrying this construct were tested. We obtained the first experimental evidence that the *neu* protooncogene has mitogenic and transforming activities only in the presence of a ligand

stimulating its tyrosine kinase activity. Various cellular and molecular biological parameters indicated that the chimeric receptor behaves very similarly to the EGFR. Also, this chimeric receptor has allowed us to compare the constitutive oncogenic and the ligand-activated nononcogenic activities of the *neu* tyrosine kinase. These models should be amenable to analysis of the corresponding receptor-binding ligands and their phosphorylation. In the future we also plan to focus on the characterization of possible differences between EGFR and *neu* signaling in more differentiated cellular backgrounds.

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# 12. Involvement of protein kinase C in the growth regulation of human breast cancer cells

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## 1. Introduction

The relationship between growth of breast cancer and endocrine regulatory mechanisms is well established [1–7]. Numerous studies provided evidence for a distinct and important role of estrogen and progesterone and their receptors (ER and PR) in the growth regulation of human breast cancer (HBC) [1–7]. At present there is no unifying hypothesis about specific alterations that lead to the initiation and progression of HBC [5–7]. However, it is clear that human breast cancer progresses from an estrogen-responsive (hormone-dependent) ER+ tumor to a more aggressive estrogen-unresponsive (hormone-independent) ER− phenotype that is less or poorly amenable to endocrine therapy [1–7]. Since only few hormone-independent HBC tumors lack the ER, the loss of the ER cannot explain the lack of antiestrogen sensitivity [1–7]. Despite many studies, the molecular mechanisms by which steroid hormones regulate the growth of both normal mammary and tumor cells remain elusive. It has been proposed that the escape from estrogen-dependent growth of HBC cells is due, at least in part, to alterations in their autocrine and/or paracrine growth mechanisms [16–23]. Consequently, disordered secretion of growth factors (GFs) from HBC cells and/or surrounding stromal and other cells may result in a permanent activation of intracellular signaling pathways contributing in the bypass of the estrogen-dependent growth control of HBC cells and hence progression of the disease.

Transduction of mitogenic signals is often coupled to protein kinases intrinsic to or associated with the receptors of GFs [8–11]. These protein kinases have been subdivided into two major groups according to their ability to either phosphorylate serine and threonine (serPK) or tyrosine (TPK) of substrate proteins [8–11]. Binding of GFs to their respective surface receptors often results either from receptor-activated tyrosine phosphorylation or receptor coupling to GTP-binding proteins in the activation of specific phospholipases that elevate intracellular levels of calcium and diacylglycerol (DG) [12–15]. A multifunctional serPK — the protein kinase C (PKC) — which has been identified as the major receptor for tumor-

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

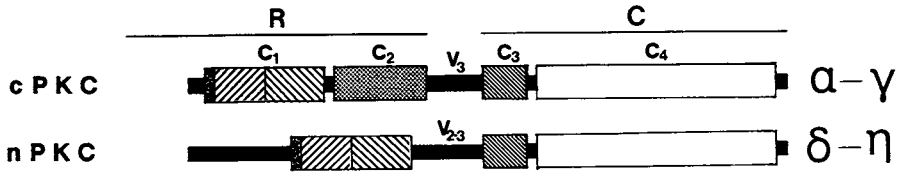


*Figure 1.* Domain structures of PKC. The domain structure of the calcium-dependent PKCs (cPKC) and the calcium-independent group of PKCs (nPKCs) are depicted. R = regulatory domain containing the two 'zinc fingers' (C1); C = catalytic domain; $V_1$ – $V_5$ = variable and $C_1$ – $C_4$ conserved regions, respectively. $C_3$ contains the ATP-binding consensus sequence. PS = pseudosubstrate; $V_2$ – $V_3$ = protease-sensitive hinge.

promoting phorbol esters (TPA), utilizes the DG and calcium to modulate various cellular functions [16–23].

Originally discovered as a phospholipid- and calcium-dependent serPK, molecular cloning revealed a family of related but distinct enzymes [16–19]. Thus far, one calcium-dependent group of enzymes (cPKCs), including the α-, β1-, β2-, and γ-PKCs, and a calcium-independent group of nPKCs, including the δ-, ε-, ζ-, and η-subtypes, have been identified (Figure 1) [16–19]. Comparison of their amino acid sequences revealed that each PKC molecule can be subdivided into large conserved and smaller highly variable subtype-specific sequences [16–19]. The protein kinase domain of PKC is confined to the C terminus and is separated by a protease-sensitive hinge region from the regulatory N-terminal domain (Figure 1) [24–26]. The latter displays two invariant cystein-rich 'zinc fingers,' with the exception of the ζ-PKC, which contain only one of these finger structures [16–19]. These cystein-rich clusters function in the binding of tumor promoters, DG, and presumably DNA [16–19,27–30]. A region that is absent in all of the members of the calcium-independent group of PKCs appears to be linked to the calcium requirement for enzyme activity [16–19,31,32].

In the absence of activators, the catalytic activity of the enzyme is maintained in an inactive state by interaction of the protein kinase domain with a short sequence designated as pseudosubstrate, located N-terminal to the first cystein-rich region [33,34]. Little is known about the cellular functions of the individual PKC subtypes. However, specific function(s) of each individual PKC subtype in various cellular responses can be anticipated due to their differential localization in tissues and cells, and to their different modes of regulation and their different substrate specificities [16–23]. The finding that tumor-promoting phorbol esters such as TPA can replace DG in the activation of PKC has provided insights into the role of this enzyme family with respect to the regulation of a variety of cellular processes, such as exocytosis, gene expression, proliferation, differentiation, and tumor promotion [16–23].

In contrast to the transient activation of PKC by DG, the activation of PKC by TPA-like phorbol esters is more protracted and results in the down-

regulation of certain subtypes of PKC by proteolysis [16–22]. Nevertheless, experimental evidence suggests that permanent activation of the PKC by either tumor-promoting phorbol esters or elevated levels of DG, as well as by overexpression of normal or mutated forms of PKC, contributes to multiple growth abnormalities and tumorigenicity of untransformed cells, presumably by altering the expression of genes that are tightly controlled by GFs in normal cells [16–23,35–42].

The finding that certain cellular responses to GFs are either unaffected or partially lost after downregulation of PKC activity has led to the assumption that mitogenic signals induced by GFs can be linked to either PKC-dependent or PKC-independent pathways [18,42]. In contrast, an obligatory step in the mitogenic response elicited by GFs is the activation of protein synthesis, which is thought to be regulated in part by multiple phosphorylations of the S6 protein located on the 40s ribosomal subunit [10,43,44]. Phosphorylation of the S6 protein invariably occurs following mitogenic stimulation of cells and is due to the activation of specific serPKs, collectively termed the *S6 kinases* (S6-PKs) [10,43,44]. Activation of S6-PKs by mitogens are believed to be a prerequisite for the initiation of protein and subsequently DNA synthesis [10,43–47]. The recent molecular cloning of multiple species of S6-PKs provided evidence for the existence of two classes of S6-PK enzymes [10,43–47]. The finding that stimulation of S6-PK activity occurs by serine/threonine phosphorylations indicates the existence of a phosphorylation cascade between the GF receptors and the S6-PKs [10,43–51]. In summary these data suggest that a single mitogen may act through multiple pathways to induce a specific cellular response. For S6 protein phosphorylation this could be through the activation of different S6-PKs or different signaling systems converging on the same S6-PK.

The aim of this review is to summarize experimental evidence of the possible involvement of PKC in the growth regulation of HBC cells, with special emphasis on its endogenous subtrates and its regulatory action on the receptor for epidermal growth factor (EGFR) and S6-PK.

## 2. Results

### *2.1. Involvement of PKC and S6 phosphorylation in the growth regulation of HBC cells*

Various aspects of HBC growth can be studied using either estrogen-responsive (ER+) or estrogen-unresponsive (ER−) HBC cell lines [52,53]. Whereas ER+ HBC cells have an absolute requirement for estradiol ($E_2$) with respect to growth in vitro and athymic nude mice, the ER− HBC are autonomous with respect to $E_2$ in vitro and in vivo [5,6,52,53]. In addition to $E_2$, various GFs, such as insulin-like growth factor I (SMC), epidermal growth factor (EGF), and the EGF homologue, α-type tumor growth

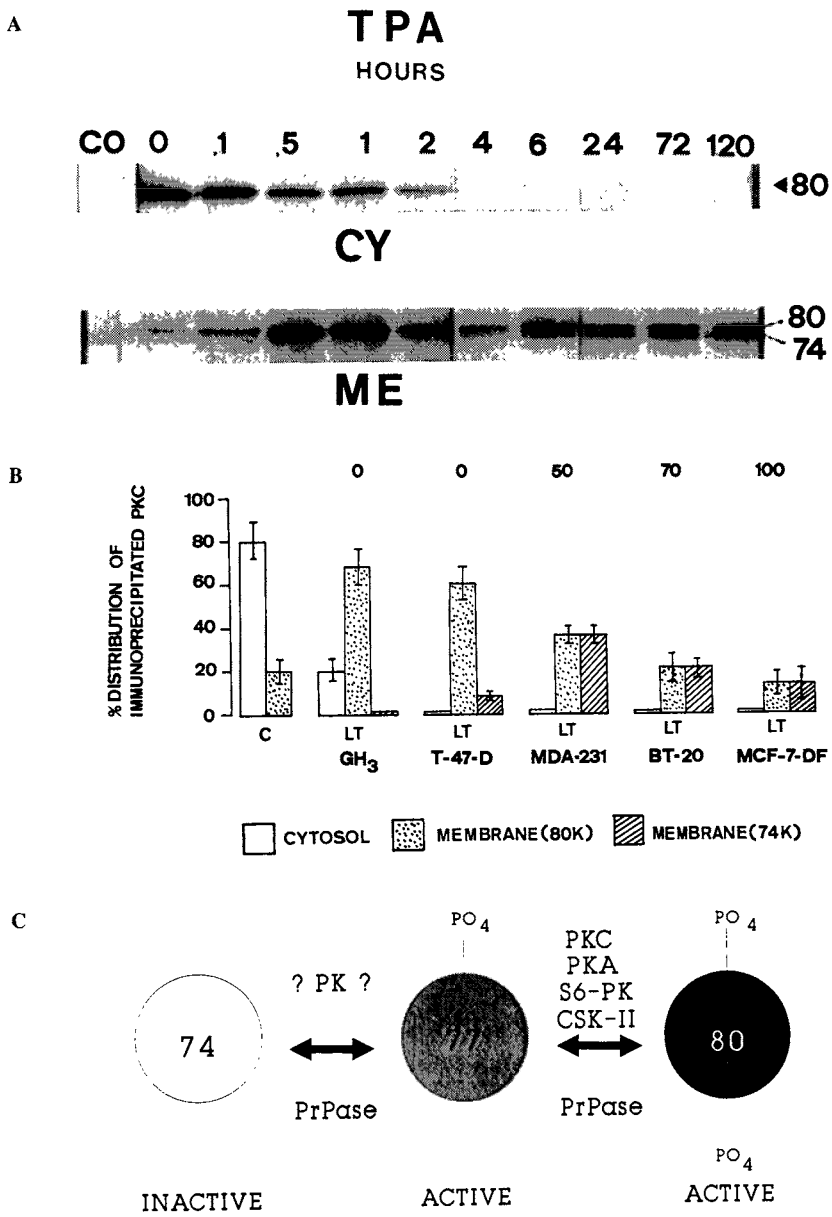


*Figure 2.* Downregulation, synthesis, and post-translational modification of α-PKC during TPA treatment. MDA-MB-231 cells were incubated with 300 nM TPA for 6–120 hours followed by [$^{35}$S]methionine labeling for the last 3 hours, and the α-PKC subtype was immunoprecipitated (IP) from cytosol (CY) and membranes (ME) as described [62]. A: Autoradiogram. IP in the presence of excess unlabeled pure PKC from cells exposed for 1 hour to 300 nM TPA (CO). B: Subcellular distribution of synthesized α-PKC. Quantitation of IP from rat pituitary $GH_3$ and various human breast cancer cell lines exposed to 300 nM TPA for 48 hours (LT), as well as their respective control cells (C). The total PKC from control cells represents 100%. Numbers

factor-α (TGF-α), are also able to stimulate growth and S6 phosphorylation of ER+ cells [54,55]. However, stimulation of S6 phosphorylation was not observed with physiological concentrations of $E_2$ in ER+ HBC cells, suggesting that GFs secreted under the influence of $E_2$ presumably function in a paracrine rather than autocrine manner [5,6,54,55]. In contrast there was no stimulation of cell proliferation and S6 phosphorylation by insulin, SMC, EGF, or αTGF in ER− HBC cells, although these cell lines contain a functional S6 kinase (S6-PK) and express receptors for EGF and SMC [54,55,57,58]. In addition, most of the ER− HBC cells displayed significantly higher growth rates in the absence of serum and GFs as compared to the ER+ HBC cells [54,55].

According to these results, stimulation of S6 phosphorylation elicited by GFs correlated with their ability to stimulate growth of the HBC cells. Furthermore these data suggest that the receptors for EGF and SMC in ER− HBC cells appear unable to transmit their signals to the S6-PK [54,55]. Whether the lack of S6-PK activation by these GFs in ER− HBC cells is due to an altered signaling of their cognate GF receptors or is eventually linked to the finding that the ER− HBC cell lines express higher levels of EGFR and PKC as compared to ER+ HBC cells, remains to be investigated [54,55,57,58]. A similar negative correlation between the levels EGFR or PKC activity and the receptors for estrogen (ER) and progesterone (PR) has also been found in biopsies of HBC patients [54,57–59]. Elevated levels of PKC activity have also been reported in specimens of primary breast tumors compared to the surrounding normal breast tissue, as well as in multidrug-resistant variants of MCF-7 cells [20]. Furthermore, clinical studies have demonstrated that the high levels of the EGFR and overexpression of the EGFR-related gene, HER-2, in human breast tumors are associated with poor prognosis [59,60]. Collectively these studies indicate that the late stage of human breast cancer is characterized by high levels of PKC, EGFR, and HER-2, as well as the lack of normal responsiveness towards estrogen and various GFs.

There is little information regarding the possible mechanistic function of PKC in human breast cancer. As determined by subtype-specific antibodies, only the α-PKC, and more recently the ε-PKC subtype, have been found to be expressed in established HBC cell lines [61–66]. Exposure of HBC cells to the tumor promoter TPA resulted in a rapid subcellular redistribution (translocation) of the α-PKC subtype from the cytosol to membranes in all HBC lines, as shown in Figure 2A [61–66]. Prolonged exposure of HBC cells to TPA resulted in the complete downregulation of the α-PKC subtype

above bars indicate percentage growth inhibition by TPA for the respective cell line. C: Hypothetical model showing post-translational phosphorylations of α-PKC. α-PKC is synthesized in an unphosphorylated 74-kDa form and is converted into the 77- and 80-kDa α-PKC by post-translational phosphorylation. Unidentified 'protein kinase C kinase' (?PK?). PKA = cAMP-dependent PK; CSK-II = casein kinase type II; S6-PK = S6-kinase; PrPase = serine/threonine-specific phosphatase(s).

activity by proteolysis and induced inhibition of growth of the various HBC cell lines to different extents [54,58,61–66]. Preliminary data indicate that the TPA-mediated translocation and downregulation of the ε-PKC subtype differs from that of the α-PKC [64]. The differential growth inhibition of the various HBC cell lines induced by TPA correlated with neither the levels of PKC and EGFR, nor with the steroid receptor content of the respective HBC cell line [54,57,58,61–66].

Interestingly, the ER+ T47-D HBC cell line that displayed the lowest levels of α-PKC and ε-PKC was not growth inhibited by TPA [54,57,61,65]. Nevertheless, removal of TPA from growth-inhibited HBC cells resulted in a gradual recovery of the α-PKC activity, which was associated with the resumption of cell growth [65,66]. None of the breast cancer cell lines responded to TPA with a growth inhibition [65,66] if the exposure time to TPA was sufficient for the translocation but not for the downregulation of PKC activity. Although TPA causes a total loss of PKC activity, all of the HBC cells continuously synthesize and accumulate in membrane fractions an enzymatically inactive α-PKC forms with molecular weights (MW) of 74 and 80 kDa (Figure 2A) [62,63].

The levels of continuously synthesized inactive membrane-bound 80-kDa α-PKC in TPA-treated HBC cells were inversely related to the extent of TPA-mediated growth inhibition of the respective HBC cell line (Figure 2B) [62]. The 74-kDa α-PKC form could be identified as the primary translation product of the α-PKC gene [62,63]. According to our findings, the α-PKC is synthesized as an unphosphorylated and presumably inactive 74-kDa form that is rapidly converted into the active 77- and 80-kDa α-PKC forms by post-translational phosphorylation (Figure 2C) [63]. The phosphorylation of the 74-kDa to the 77-kDa form is probably achieved by a specific 'PKC-kinase' and is significantly protracted in HBC cells treated with TPA [63]. In contrast, phosphorylation of the 77-kDa to the 80-kDa α-PKC can occur by either PKC a process termed *autophosphorylation* or by various other serPKs (Figure 2C) [63]. Therefore it appears that the accumulation of inactive 74- and 80-kDa α-PKC forms in membranes of HBC cells exposed for a prolonged time period to TPA is due, in part, to protracted post-translational processing of the 74-kDa α-PKC precursor [62]. These data give strong evidence that the molecular mechanisms responsible for the individual growth responses of HBC cell lines towards TPA appear to reside distal to the downregulation of the α-PKC.

### *2.2. Intracellular targets of PKC in HBC cells*

The addition of TPA resulted in the rapid dose-dependent reduction of EGF binding in all HBC cell lines, albeit by different mechanisms [57,58]. TPA reduced mainly the number of the low-affinity EGFRs in the ER− HBC cell lines, whereas in ER+ HBC cell lines the high-affinity binding to the EGFRs was reduced by TPA [57,58]. From recent reports it is well

established that in a variety of cell types TPA abolishes high-affinity EGF binding, reducing its intrinsic EGF-dependent TPK activity and enhancing EGFR internalization [67,68]. These multiple effects of TPA on EGFR, designated *transmodulation* of the EGFR, have been attributed to the activation of PKC [67,68]. Recent studies by site-directed mutagenesis have shown that phosphorylation of $Thr_{654}$ on the EGFR is only responsible for the inhibition of the EGF-dependent TPK activity but not the TPA-mediated loss of the high-affinity EGFR [67]. Loss of the high-affinity EGF binding by TPA also seems not to be associated with the other three major serine/threonine phosphorylation sites on the EGFR [67]. Thus PKC may exert its actions on the apparent high-affinity EGF binding by either phosphorylation of minor sites on the EGFR or through indirect means, presumably involving proteins that associate with the EGFR. Nevertheless, rapid desensitization of EGFR signaling through PKC-activated by the DG generated via the EGF-dependent tyrosine phosphorylation and stimulation of the γ-type phospholipase C provides an efficient negative-feedback signal for the termination of the EGF action [12–15,67,68].

Furthermore, it should be noted that TPA reduces only transiently the binding of EGF to its receptor in HBC cells (Figure 3A). The reduction of EGF binding upon prolonged exposure of HBC cells to TPA was followed by the almost complete reappearance of EGFRs on the surface of HBC cells and coincided with the downregulation of PKC activity, as demonstrated in Figure 3A. Recovery of the EGFR is presumably due to enhanced de novo synthesis of the EGFR initiated by the activation of PKC, as evidenced by the accumulation of EGFR transcripts in TPA-treated HBC cells (Figure 3A). The EGFR population synthesized following prolonged TPA treatment displayed binding affinities to EGF similar to those found in untreated HBC cells. Although downregulation of PKC activity in ER+ HBC cells blocks the stimulation of S6-PK activity by TPA, it leaves the activation of S6-PK by EGF — as well as by TGF-α, insulin, or SMC — unaffected (Figure 3B) [54]. Taken together, these result suggest that transmodulation of the EGFR by TPA is lost following depletion of PKC activity in HBC cells (Figure 3).

Neither EGFR nor other GFs were able to stimulate the proliferation of the ER+ HBC cell lines made deficient of PKC activity by prolonged TPA treatment [58]. The apparent contradiction that EGF is able to stimulate S6 phosphorylation without stimulation of cellular growth in ER+ HBC depleted of PKC may be explained by the finding that EGF induces an early and a late phase of S6-PK activity [50]. In contrast to EGF, TPA stimulated only the late phase of S6-PK activity. When cells were depleted of PKC by long-term TPA treatment, the stimulation of S6-PK by TPA and late-phase stimulation of S6-PK by EGF were lost, while the early phase of EGF-dependent S6-PK activation remained unaffected [50]. The apparent loss of the late-phase stimulation of S6-PK activity by EGF resulted in a substantial decrease in the phosphorylation of the S6 protein, protein synthesis, and cellular proliferation [50]. Thus PKC appears to be a prerequisite for

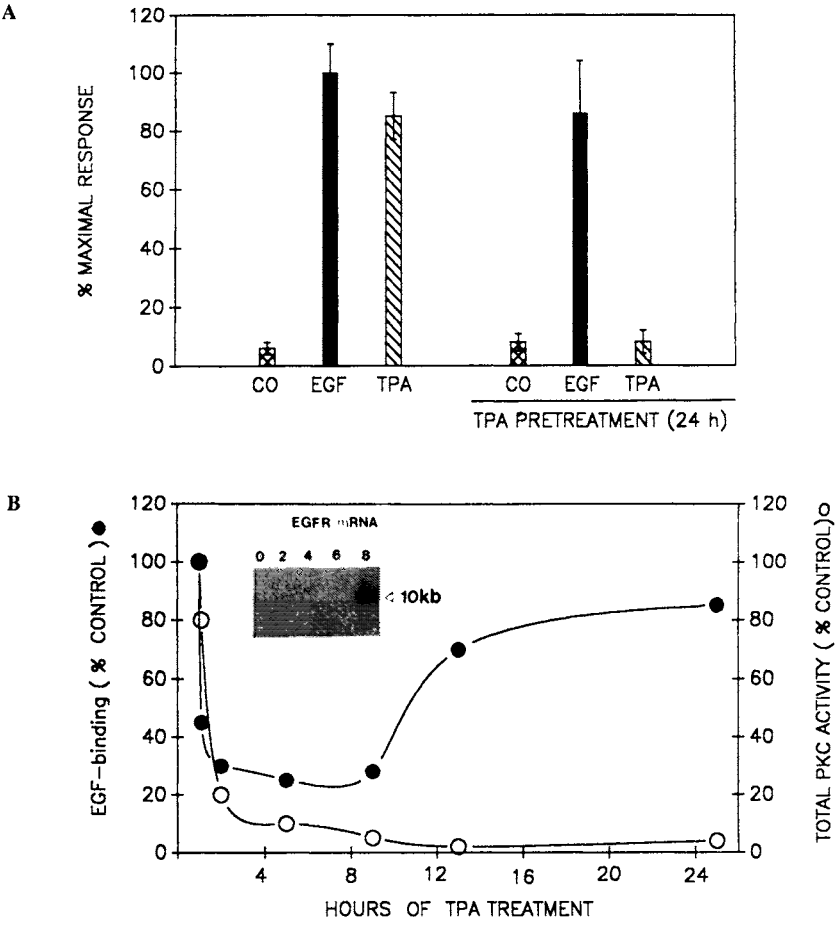


*Figure 3.* Effects of TPA on the EGFR of HBC cells. A: S6-PK activation by EGF and TPA. Untreated ZR-75-1 cells or ZR-75-1 exposed for 24 hours with 300 nM TPA were exposed to either 300 nM TPA or 50 nM EGF for 30 minutes. Stimulation of S6 phosphorylation was quantified as described [55]. B: Time course of the downregulation of α-PKC activity and EGF binding in ZR-75-1 cells. EGF binding and PKC activity were determined as decribed in Fabbro et al. [57]. Insert: Northern blot analysis of the EGFR mRNA using the 1.8-kb *Eco*RI fragment derived from pHER-A64-1 was performed as decribed in Wartmann et al. [41]. Only the 10-kb EGFR mRNA is shown.

transmitting the mitogenic signal elicited by EGF to the S6-PK. In contrast to the transient transmodulation of the EGFR, PKC activation by TPA also resulted in a permanent loss of progesterone binding to the PR without affecting the binding of estrogen to its receptor in ER+ HBC cells [58]. In this context it is noteworthy that the addition of EGF to ER+ HBC cells yielded in a similar downregulation of the progesterone receptor content [69].

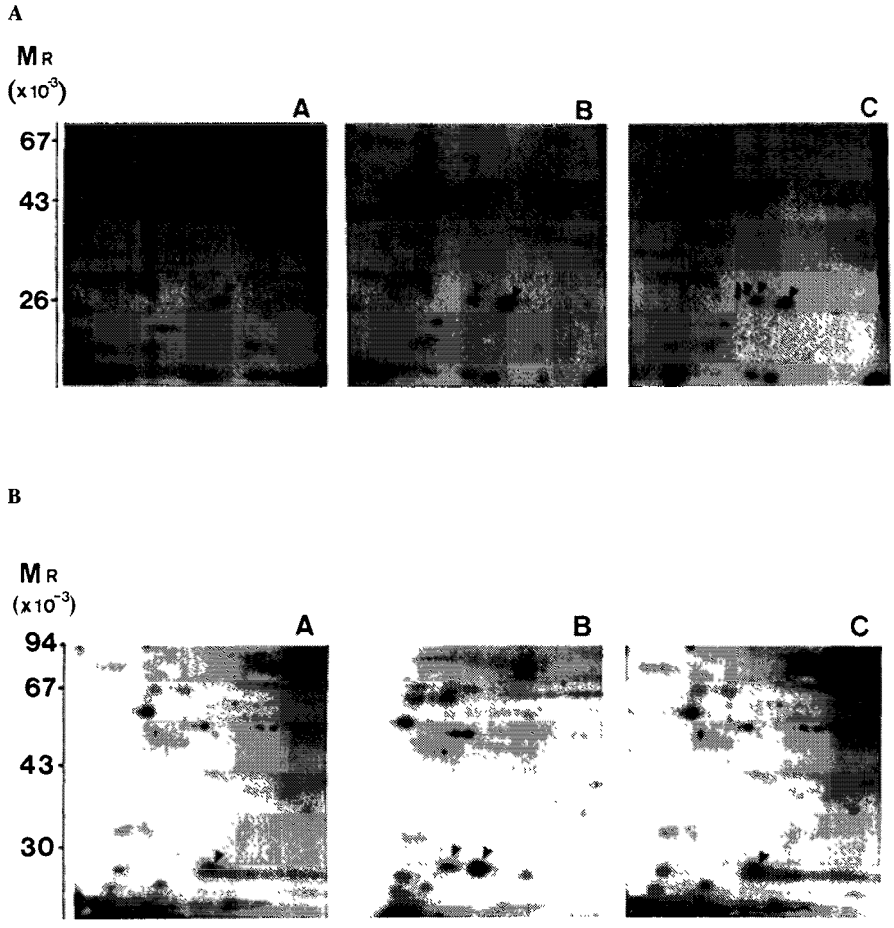


*Figure 4.* Effects of TPA and stress inducer on the phosphorylation of the hsp27 of HBC cells. A: Effects of TPA on hsp27 phosphorylation. HBL-100 HBC cells were exposed for 24 hours to TPA (100 nM). TPA-treated cells (C) or control cells (A,B) were labeled with $^{32}P$, followed by incubation with (B,C) or without 100 nM TPA (A) for 30 minutes. Phosphoproteins were resolved by two-dimensional gel electrophoresis, as described in Regazzi et al. [70]. Arrows denote the two isoforms of hsp27. B: Effects of stress inducers on hsp27 phosphorylation. HBL-100 HBC cells, untreated (A,B) or exposed for 24 hours to 100 nM TPA (C), were incubated in the absence (A) or presence (B,C) of 100 μM $NaAsO_2$. Phosphoprotein was analyzed as decribed in Regazzi et al. [70].

Addition of TPA to HBC cells also led to the phosphorylation of two abundant cytosolic phosphoproteins of 27 kDa (heat shock protein p27 [hsp27]) and 17 kDa (pp17), as illustrated in Figure 4A. Two isoforms of the hsp27 displaying isoelectric points of 5.5 (hsp27a) and 5.0 (hsp27b) were observed, both of which are phosphorylated in response to TPA, exogen-

ously added bacterial phospholipase C, calcium ionophore, or fetal calf serum; whereas insulin, EGF, or dibutyryl-cAMP did not change the phosphate content of hsp27 [70]. The time course of hsp27 phosphorylation closely paralleled the rapid TPA-induced subcellular redistribution and downregulation of PKC activity [70]. According to these and other criteria, the hsp27 appear to be specific substrates for PKC in HBC cells.

Suprisingly, exposure of HBC cells to stress inducers, such as arsenite or cadmium, also increased the $^{32}P$ incorporation into both hsp27 species that were comparable or higher than those obtained with TPA [70] (Figure 4B). The amounts of $^{32}P$ incorporated into hsp27 were proportional to the amounts of the hsp27 polypeptides synthesized following exposure of HBC cells to stress inducers [70]. This is in contrast to TPA, which does not alter the synthesis of hsp27. If HBC cells were made deficient of PKC by prolonged incubation with TPA, the subsequent addition of TPA or phopholipase C did not induce the phosphorylation of the hsp27 (Figure 4A) [71]. Phosphorylation and synthesis of hsp27 during stress treatment were not affected after downregulation of PKC activity (Figure 4B) [71].

These data raise the possibility that some of the major PKC substrates in HBC cells are members of the heat shock protein family whose phosphorylation is not mediated by PKC during the stress response. It is known that exposure of cells to increased temperature or to stress inducers, such as heavy metals, arsenite, or cytotoxic drugs, results in a rapid synthesis of a set of highly conserved proteins, which are referred to as heat shock or stress proteins [72]. Among this group of proteins there is a family of small polypeptides with 27 kDa MW that are phosphorylated on serine residues and that display properties identical to those oberved for the hsp27 of HBC cells [73–76]. Although considerable work has provided details regarding the various post-translational modifications of hsp27 during stress conditions, their physiological role is not yet clear. The synthesis of the hsp27 proteins has been recently shown to be induced by $E_2$ in ER+ HBC cells [77], which indicates that under physiological conditions the hsp27 may be involved in the estrogen-dependent growth regulation of HBC cells [77]. The finding that primary tumors of HBC patients with poor prognosis revealed high levels of immunodetectable hsp27 was interpreted as an indication of the progression of HBC cells towards a multidrug-resistant phenotype [77].

### *2.3. PKC as potential therapeutic target*

There is an urgent need for the development of novel antiproliferative agents with better selectivity and tolerability, since most of the current therapies have no significant impact on the growth of tumors of epithelial origin. Nonsteroidal triphenylethylene (TPE), such as tamoxifen and its derivatives, which inhibit the proliferative actions of a biologically active estrogen receptor, are often clinically applied to inhibit breast cancer cell

growth [80]. It is accepted that TPE prevents the binding of $E_2$ to the ER [80,81]. However, this mechanism of action appears not to be exclusively responsible for the antiproliferative action of TPE [81].

TPE derivatives have been shown to inhibit PKC, and their estrogen-irreversible cytotoxic effects of TPE antiestrogens have been reported to correlate with their inhibitory potencies against PKC, but not with their affinities for the receptors for estrogen or antiestrogens [20]. Therefore, PKC may represent a valuable pharmacological target for the development of protein kinase inhibitors with antiproliferative properties suitable for HBC therapy. Such a conception is further supported by the finding that protein kinases other than PKC are found to be elevated in the late stage of HBC, such as EGFR and HER-2 [54,59,60]. However, most of the compounds that have been shown to inhibit PKC are either not very specific for PKC, display inhibitory activity only in a micromolar range, similar to that found for TPEs, or show marginal effects on the growth of mammalian cells [82].

The recently discovered staurosporine, an alkaloid of microbial origin, has been found to be one of the most potent inhibitors of protein kinases displaying potent antiproliferative activity in vitro [83]. Unfortunately, staurosporine shows little selectivity towards a variety of serPKs or TPKs [84,85]. Derivatives of staurosporine display a better selectivity towards PKC inhibition [84]. Among them, a selective CGP 41 251, as well as an inactive analog (CGP 42 700), have been identified [84,85]. Although CGP 41 251 was less potent than staurosporine (CGP 39 360), it was more selective in inhibiting PKC activity in vitro [84,85]. In this context it should be noted that CGP 41 251 did not display selectivity against either α-, β-, or γ-PKC subtypes [85].

Inhibition of PKC activity by staurosporine or CGP 41 251 is due to competition with ATP without affecting phorbol ester binding [83–85]. Staurosporine and CGP 41 251 inhibited not only cellular PKC activity, but also TPA-induced formation of hydrogen peroxide by human monocytes and cell growth, with potencies comparable to those obtained with purified PKC (Figure 5A) [83–85]. In addition, staurosporine not only inhibited the TPA-dependent phosphorylation of the stress protein hsp27 but also the basal phosphorylation of a phosphoprotein band with a MW of 16 kDa (pp16) whose phosphorylation is not stimulated by TPA in HBC cells [86]. Although CGP 41 251 was less potent in inhibiting the TPA-dependent hsp27 phosphorylation, it had no effect on the basal phosphorylation of pp16 [86]. Furthermore, only staurosporine (CGP 39 360), but neither CGP 41 251 nor the inactive analog CGP 42 700, caused a translocation of PKC to membranes of MDA-MB-231 cells or induced a calcium-dependent binding of purified PKC holoenzyme to erythrocyte inside-out vesicles, as shown in Figure 5B [86,87].

The translocation of PKC induced by staurosporine was not comparable with that found by TPA [86]. Staurosporine induced a slower but permanent

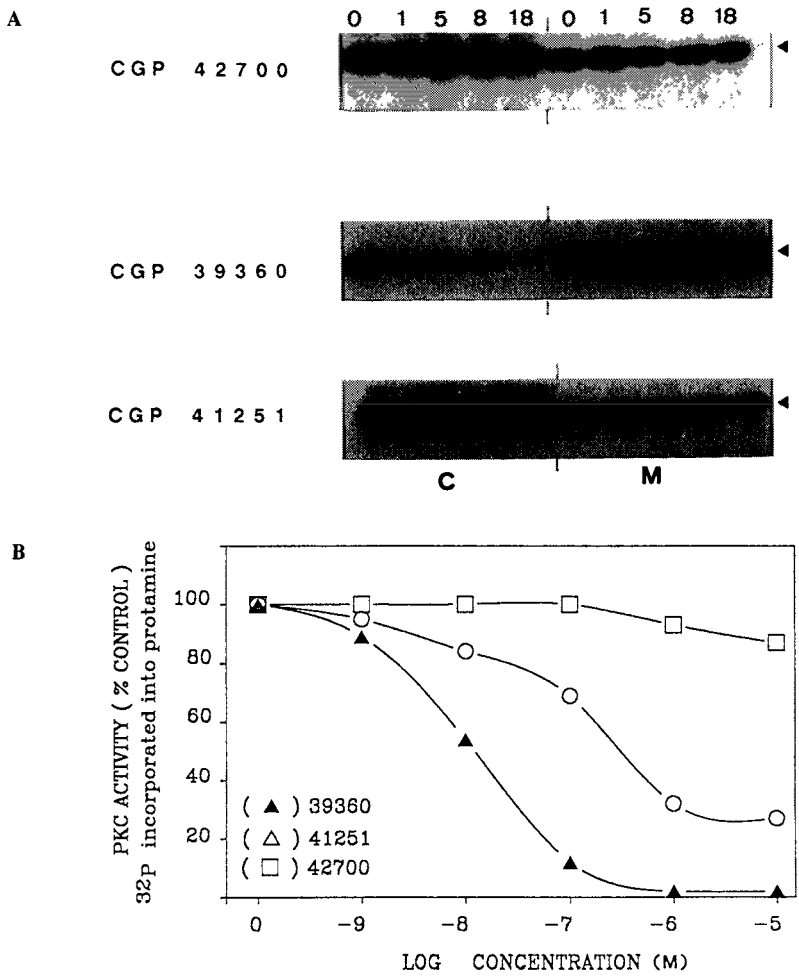


*Figure 5.* Effects of staurosporine and derivatives on cellular PKC. A: Effects of staurosporine and derivatives on cellular PKC activity. MDA-MB-231 cells were treated with increasing concentrations of CGP 39,360 (staurosporine), CGP 41,251 or CGP 42,700 for 5 minutes. PKC activity in the cytosolic fraction was determined using protamine [86]. B: Effects of staurosporine on immunodetectable PKC. MDA-MB-231 cells were exposed for the indicated times to 1 μM CGP 39,360, 1 μM CGP 41,251, and 10 μM CGP 42,700. Western blot analysis of the cytosolic (C) and membrane (M) fractions of MDA-MB-231 cells was performed as described in Borner et al. [61] and Fabbro et al. [86].

translocation of PKC to membranes without causing downregulation of PKC (Figure 5B). These data suggest that staurosporine may have additional effects on PKC as well as on cellular responses not mediated by PKC. No apparent selective antiproliferative effects of CGP 41 251 nor stauropsporine were observed among the various cell lines tested [82,84]. Nevertheless, compared to staurosporine, CGP 41 251 showed an increased therapeutic

window when tested for antitumor effects in tumor-bearing animals [82,84]. The higher selectivity of CGP 41251 with respect to inhibition of purified and cellular PKC activity resulted in an increased compound tolerability and an increased antitumor effect at equally tolerated doses. The non-inhibitory activity of CGP 41251 against protein kinases other than PKC, such as S6-PK, the TPK of the EGFR, or the cAMP-dependent protein kinase, did not lead to a loss of antitumor effects, indicating a higher therapeutic index of CGP 41251 compared to staurosporine [84].

## 3. Conclusions

The pathopysiological role of secreted trophic substances in the neoplastic progression of HBC [5–7] is suggested by various investigations, which indicate that growth regulation of mammary tumor cells is not only limited to the presence of ER but also to the presence of GFRs [59,60]. In addition, it is well established that constitutive activation of protein kinases intrinsic to, or associated with, GFRs confers multiple growth abnormalities and tumorigenicity to untransformed cells [8–11,18–22]. Induction of cellular growth by GFs has also provided ample evidence for the role of the proto-oncogenes c-*myc* and c-*fos* in cellular proliferation [21,22,88,89]. Expression of c-*myc* and c-*fos* are regulated by GFs and $E_2$ in the ER+ HBC cells, whereas in the ER− HBC cells the levels of c-*myc* and c-*fos* are either constitutively high or are less inducible [90,91]. The inducibility of these proto-oncogenes by GFs in ER+ HBC cells agrees with their growth stimulation and the S6-PK activation induced by these GFs [54,55]. The loss of GF-induced S6 phosphorylation in ER− HBC cells is not due to a lack of a functional S6-PK, implying that in these cells GFs are unable to transmit the mitogenic signal to the S6-PK. Whether the inability of GFs to stimulate S6 phosphorylation in ER− HBC cells is due to the constitutive activation of their cognate receptors, leading to a permanent activation of c-*fos* and c-*myc*, remains to be investigated.

However, in this context it is of interest to note that ER− HBC cell lines and primary tumors of HBC patients with poor prognosis often display high levels of HER2, EGFR, and PKC activity [54,59–61]. It is not known how HBC cells acquire high levels of EGFR, HER2, and PKC activity. In a variety of cells and malignant diseases, the overproduction of the EGFR is due to gene amplification, enhanced transcription, or retarded metabolic turnover [92–94]. However, the high levels of EGFR in HBC cells are very unlikely to be due to gene amplification, as demonstrated for the EGFR-like HER2, whose ligand has been recently identified [59,60,95]. High levels of EGFR have been reported to facilitate growth and tumor formation only when an autocrine loop involving the EGF or the EGF homologue TGF-α was operative [96,97]. This may explain the finding that overexpression of TGF-α in the ER+ MCF-7 cell line, which displays low levels of EGFR, is not sufficient to bypass the estrogen requirement of this cell line [98]. These

data indicate that high levels of EGFR and HER2 may represent a step by which the estrogen requirement for growth may be bypassed by autocrine or paracrine mechanisms in HBC cells. Interestingly the high levels of EGFR correlated with the high levels of PKC in a variety of HBC cells and tumors [54,57,58]. PKC is known to modulate not only the binding of EGF or TGF-α to the EGFR, but also the synthesis of EGFR [58,67,68,99,100]. Whether the high levels of EGFRs displayed by the late stage of breast cancer may be causally related to the high levels of PKC remains to be investigated.

The activation of PKC by the tumor promoter TPA induces an array of cellular responses in HBC cells [54,55,58,61–66]. In contrast to the majority of cells lines in which TPA stimulates growth, exposure of HBC cells to TPA resulted in a reversible growth inhibition to different extents [58,65]. According to our data, the molecular mechanisms responsible for the individual growth responses of HBC cell lines towards TPA appear to reside distal to the downregulation of the α-PKC [62,63,66]. The addition of TPA to HBC cells resulted in a transient reduction of EGF binding [58]. Following downregulation of PKC by TPA in the ER+ HBC cells, the EGFRs appeared to be functional with respect to the S6-PK activation induced by EGF [54]. The finding that ER+ HBC cells deficient in α-PKC after prolonged TPA treatment could no longer be growth stimulated by EGF suggests that PKC plays a central role in transmitting the mitogenic stimulus to the S6-PK.

Activation of PKC by TPA resulted in the phosphorylation of hsp27 in all HBC cell lines tested [70,71]. Our results also indicate that hsp27 are phosphorylated by PKC only during receptor-mediated stimulation of the cells, but not during the stress response [71]. The protein kinase responsible for the phosphorylation of the hsp27 during the stress response has not yet been identified. Although considerable work has provided details regarding the various post-translational modifications of hsp27 during stress conditions, their physiological role is still not known. Although the various forms of hsp27 are localized in the cytoplasm, they seem to translocate partially to the nucleus during stress treatment [76]. TPA and serum factors increase the phosphate content but neither the synthesis nor the redistribution of these proteins into the nucleus [71]. Thus the family of hsp27 may, albeit by different mechanisms, be involved in the stress response as well as in processes regulating normal growth.

The recent finding that synthesis of hsp27 appears to be regulated by estrogen under physiological conditions and that high levels of hsp27 in primary tumors of HBC patients appear to be associated with poor prognosis suggests an important role of hsp27 in the normal growth and progression of the HBC cell [77]. Most notably, overexpression of hsp27 in drug-sensitive cells has been reported to confer resistance to cytotoxic drugs [77]. The emergence of drug-resistant cells during chemotherapy is one of the major problems in cancer treatment. Multidrug resistance is commonly

associated with the overexpression of the membrane-spanning product of the MDR-1 gene, the multidrug transporter [78]. In this context it should be emphasized that the expression of the human multidrug transporter mRNA can also be induced by stress (arsenite) or heat shock through heat-shock-responsive elements that are present in the regulatory region of the MDR-1 gene [79]. Since expression of the MDR-1 gene can also be induced by stress inducer or heat shock, and both hsp27 and the multidrug transporter have been reported to be substrates for PKC, these findings may provide a possible mechanistic link among the high levels of PKC, hsp27, and the multidrug-resistant phenotype of HBC cells [20,70,71,78,79]. Therefore these data suggest that the overexpression of hsp27 may be correlated with a multidrug-resistant phenotype during the progression of HBC cells.

The finding that protein kinases such as PKC, EGFR, and HER2 are elevated in the late stage of HBC indicate that these protein kinases may represent a valuable pharmacological target for the development of compounds with antiproliferative properties suitable for HBC therapy. While searching for selective PKC inhibitors, a selective staurosporine derivative with in vitro antiproliferative effects was identified [82–86]. Compared to staurosporine, the CGP 41 251 displayed an increased therapeutic window when tested for antitumor effects in tumor-bearing animals [82,84]. The higher selectivity of CGP 41 251 with respect to the inhibition of cellular PKC activity resulted in an increased compound tolerability and an increased antitumor effect at equally tolerated doses [82,84]. This compound (CGP 41 251) and other derivatives selective for PKC, EGFR, or HER2 may therefore prove to be useful for the therapy of breast cancer. There is no doubt that protein kinases, such as PKC and S6-PK, as well as the tyrosine protein kinases of certain GF-Rs, appear to play a key role in the signal transduction of HBC cells. Therefore, they appear to be valuable pharmacological targets for the development of agents designed for better management of human breast cancer.

## Acknowledgments

We thank Dr. A. Ullrich for the pHER-A64-1 probe. These studies have been supported by the Swiss National Foundation grant no. 3100-26523.89 and by the Swiss Cancer League grant no. FOR.341.86.1, and a grant from Ciba-Geigy.

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# 13. Tyrosine kinase receptor — nuclear protooncogene interactions in breast cancer

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## 1. Introduction

Breast cancer afflicts 1 in 9 women in North America. It is characterized by a strong dependence on intact ovaries for onset and progression in women of all ages, but it is primarily a postmenopausal disease. This appears to be because prolonged exposure of the gland to ovarian hormones is required, but the disease is usually associated with a long latency of 20 years or more [1]. Excessive proliferation, dedifferentiation, genetic mutability, and metastases characterize the disease when it is clinically manifest. It is not yet known what defects occur early in the disease process, but ovarian hormones appear to be critical. The proliferation of the normal gland predominates in the luteal phase of the menstrual cycle, implicating an interaction of both estrogen and progesterone, which are present in this phase. In premenopausal disease, estrogen appears to be the ovarian hormone of primary importance. In postmenopausal breast cancer, when blood levels of estrogen and progesterone are extremely low, breast tumors appear to arise under control of other factors, among which are estrogen synthesized peripherally by aromatase and sulfatase mechanisms. Likely candidates for additional factors regulating breast proliferation and tumorigenesis are locally acting growth factors, genetic alterations of protooncogenes, and aberrant interactions of stromal and epithelial compartments [2]. A very interesting observation is that of the most common oncogene activations in breast cancer (c-*erb*B-2 and c-*myc* oncogene amplifications), c-*myc* amplification is associated with postmenopausal breast cancer. While c-*erb*B-2 amplification is associated with poor prognosis of breast cancer, irregardless of menopausal status, c-*myc* is not of strong prognostic significance. This suggests that c-*myc* may function in premalignant changes and early in tumor progression and that c-*erb*B-2 might be particularly important later in the progression [3]. This article will focus on the possible mechanisms of action of c-*myc* in early stages of malignancy. We will address the hypothesis that c-*myc* may act, in part, by synergistic transforming interaction with growth-factor receptor tyrosine-kinase activities. This mechanism will be contrasted with that of c-*erb*B-2 amplification, which may

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

act on its own, in the absence of c-*myc* amplification, to drive other aspects of the cancer phenotype later in malignant progression.

## 2. Growth factors: Regulation of proliferation and interaction with estrogen and progesterone

Estrogen and progesterone are intimately associated with breast epithelial proliferation, tumorigenesis, and malignant progression. Thus, recent studies have begun to address the mechanisms of action of these two ovarian steroids. Many investigators are examining defective or overexpressed growth regulatory genes (oncogenes) and locally acting polypeptide hormones (growth factors) as mediators and modulators of steroid action in breast cancer [4,5]. One class of growth factors under study includes the transforming growth factors. Though probably a misnomer, they derive their names from their ability to reversibly induce the transformed phenotype (anchorage-independent growth) in certain rodent fibroblasts. They are locally acting polypeptide hormones that were initially found to be synthesized and secreted by a variety of retrovirally, chemically, or oncogene-transformed human and rodent cell lines [4,5]. They now are known to have widespread distribution and quite varied functions.

Two major classes of structurally and functionally distinct transforming growth factors are TGF-α and TGF-β. TGF-α and TGF-α-like peptides are members of a multiple-species family (of cellular and viral members ranging in apparent molecular masses from 6 to 43 kDa. All members are disulfide-linked monomeric growth factors that bind to the cell surface receptor known as the epidermal growth factor (EGF) receptor [7–11]. The EGF receptor and the receptors for growth-stimulatory growth are transmembrane molecular factors that function through regulation of a tyrosine kinase domain in their cytoplasmic region. The TGF-β family consists of at least five related gene products, each apparently forming 25-kDa homodimeric and heterodimeric species. There appears to be a complex pattern of interaction of these species with the TGF-β receptors, which have been described as three species of different molecular weights that have not yet been fully characterized [12,13]. TGF-β, and more recently TGF-α, have been found in the urine and pleural and peritoneal effusions of cancer patients [14–16]. These growth factors have also been observed in many normal tissues [12,13]. Treatment of both normal and malignant epithelial tissue with TGF-β of all subtypes tested generally has a growth-inhibitory and sometimes differentiating effect [12,13]. In contrast, TGF-α is generally growth stimulating and sometimes prevents differentiation.

At least two other classes of monomeric, but disulfide-linked, growth factors are also relevant: insulin and insulin-like growth factors (IGF-I and IGF-II) and their binding proteins and four receptors comprise one group. The second group is the fibroblast growth factors (FGF) family of at least

seven members with at least four receptors [4,6]. Whereas insulin is an endocrine hormone, IGF-I and IGF-II are thought to have more localized synthesis and function. A more recently described growth factor, mammary-derived growth factor 1 (MDGF-1), has been found in human milk and in conditioned medium from human breast cancer cell lines [17–19]. This glycosylated, monomeric, and non-disulfide-linked 62-kDa growth factor may also play a role in the growth regulation of normal and malignant human mammary epithelium through its recently described tyrosine-kinase-associated receptor. It has been hypothesized that transformation of cells from normal to malignant may directly result from increased production of growth-stimulatory factors, decreased production of growth-inhibitory substances, or altered responsiveness to either or both transforming groups of growth factors [6]. This represents an interesting and testable possibility, but malignant progression of breast cancer during its clinical course usually results in highly malignant, metastatic disease in its final stage, with no discernable direct growth regulation by any hormones or growth factors in the patient.

An important perspective in understanding pathways of growth control in human neoplastic cells is knowledge of growth regulation of the normal cells from which the cancer derived. To date, this area of investigation in epithelial cells has lagged behind studies of the influence of growth factors in cancer due to the difficulties involved in the culture of normal epithelial cells. However, the recent development of specialized serum-free culture conditions has facilitated the study of growth regulation in normal human keratinocytes [20], normal human bronchial epithelial cells [21], and normal human mammary epithelial cells [22,23]. Though basal, 'stem cell'-like human mammary epithelial cells may now be cultured in vitro, it is not yet clear that this cultured cell subtype is of the lineage or differentiation type(s) that would give rise to any of the several types of breast cancer in a woman. For example, neither receptors for estrogen and progesterone, nor for luminal keratin 19, have been demonstrated in these cells, though the majority of clinical breast tumors possess at least one of these markers [22–24].

Studies on steroid-growth factor interactions in human mammary tissue have been generally restricted to the malignant epithelium. In hormone-responsive human breast cancer cells, growth stimulation by estrogen is accompanied by an increase in growth-stimulatory TGF-α or EGF and EGF receptor production [7,25,26], whereas growth inhibition of hormone-responsive breast-cancer cell lines by an antiestrogen is paralleled by augmented secretion of growth-inhibitory TGF-β [27]. Similar effects have been observed with progestins: TGF-α, EGF, and the EGF receptor are induced, while TGF-β is inhibited (antiprogestins having the opposite effect) [28–30]. In hormone-independent breast cancer cell lines, however, many of these growth factors, as well as many other growth-regulatory peptides, are constitutively produced [31–33]. These results are consistent with, but do

not prove, a role for growth factors in the expression of a more malignant phenotype and escape from normal hormonal control.

### 3. Multiple roles for TGF-α and EGF in mammary proliferation, carcinogenesis, and tumor growth

It is of note that milk, the natural secretory product of the mammary epithelial cell, is an extraordinarily rich source of growth factors, including members of the TGF-α, TGF-β, insulin, and MDGF families. This suggests a possible functional roles for newborn as well as for mammary gland physiology [34]. EGF and the closely related TGF-α appear to be important regulators of both the proliferation and differentiation of the mouse mammary gland in vivo and of mouse mammary explants in vitro [35,36]. EGF or TGF-α is also a required supplement for the clonal anchorage-dependent growth, in vitro, of normal human mammary epithelial cells [37]. However, although human breast cancer cells do not require exogenous EGF for continuous growth, many breast cancer cell lines retain receptors and growth-stimulatory responses to exogenous TGF-α and EGF [38,39].

Biologically active, EGF/TGF-α-like proteins can be extracted from mouse mammary glands undergoing lobuloaveolar growth during midpregnancy [35]. TGF-α can produce qualitatively the same biological effects as EGF in mouse mammary explants and cultured human and mouse mammary epithelial cell lines, but it appears to be more effective [35,40]; TGF-α and EGF can both induce lobuloalveolar growth. The localizations and possible functions of TGF-α and EGF in mouse mammary development have been recently studied [46]. Different patterns were observed depending upon the individual growth factor and the stage of development, ranging from virgin to midpregnant to midlactating. TGF-α and EGF mRNA transcripts were both detected in virgin and midpregnant glands, but during lactation only EGF was expressed. Using immunohistochemistry, TGF-α was localized in the proliferating, epithelial cap-cell layer of growing terminal endbuds and in stromal fibroblasts at the base of the terminal endbuds. This strongly suggested a close association of TGF-α with proliferation. In contrast, EGF was localized in the inner layers of the terminal endbud and in secretory luminal ductal cells. This strongly suggests a close association of EGF with secretion and lactation [2]. A functional role of EGF and TGF-α has been demonstrated in vivo in this system with small-pellet implants of the growth factors. EGF or TGF-α pellet implants stimulated endbud growth in regressed glands (following ovariectomy). This response could not be obtained with insulin [46,47].

An additional study detected TGF-α mRNA in mammary epithelium and some stromal cells by in situ hybridization during the proliferative, lobuloalveolar development state of rodent and human pregnancy [41].

TGF-α mRNA and protein, and EGF receptor are detected in vitro in proliferating human mammary epithelium but are very low in resting epithelial organoid remnants derived from reduction mammoplasty tissue [42,43]. The TGF-α acts as an autocrine growth factor in normal human mammary epithelial cells in mass culture; an anti-EGF receptor antibody reversibly inhibits proliferation [43].

A new member of this growth-factor family, termed *amphiregulin* [44], has also been discovered in a breast-cancer cell line treated with a tumor promoter, but its exact physiological role in normal and malignant proliferation remains to be determined. Paradoxically, it appears to inhibit breast tumor cells but not normal cells in vitro [51]. Two other members of this growth factor, a heparin-binding EGF-like macrophage-derived factor [48] and a human embryonal carcinoma factor called *cripto* [49], have not yet been examined in breast tissue. Also, a 30-kDa factor that binds EGF receptor and c-*erb*B-2 has been described but not sequenced [50].

TGF-α has been directly implicated as a modulator of mammary epithelial transformation in a number of studies. Overexpression of TGF-α following transfection of a human TGF-α DNA expression vector into the immortal, but nontumorigenic mouse mammary epithelial cell line NOG-8 led to its capacity for anchorage-independent growth [52]. Another study utilized MCF-10, a newly described, spontaneously immortalized human breast ductal epithelial cell line, as the recipient for the TGF-α gene. This cell line, which is negative for estrogen and progesterone receptors but contains a high level of EGF receptors, was also transformed by TGF-α transfection [53]. In contrast, TGF-α transfection into MCF-7 cells that have low levels of EGF receptor does not confer a significant growth advantage in vitro or in vivo [54].

In studies of human breast cancer biopsies, TGF-α mRNA and protein were detected in 70% or more of the specimens [7,55] and in approximately 30% of benign breast lesions [56]. Immunoreactive TGF-α has been found in fibroadenomas and 25–50% of primary human mammary carcinomas [25,57], and an EGF-related protein of 43 kDa has been recently isolated from breast cancer patient urine [58]. Perhaps detection of TGF-α/EGF in tumor biopsy serum or urine will eventually be found useful in early detection in determining prognosis or tumor burden. While it is possible that TGF-α is involved in tumor growth regulation, it has also been shown that TGF-α itself can induce ascitic fluid [59] and contribute to angiogenesis, demoplasia, and immunosuppression [4–6].

In human breast cancer cell lines in vitro, clear evidence of significant autocrine growth control by the TGF-α-EGF receptor system has only been seen in the hormone-independent MDA-MB-468 cell line, a line with high TGF-α expression and an amplified EGF receptor [60]. Such studies would appear to have implications for developing novel therapeutic strategies. However, aside from the few percent of breast cancers overexpressing EGF receptor by such a gene amplification, this growth factor receptor system

The FGFs and IGFs may also play a role in mammary proliferation and cancer. Although FGF receptors are in normal mammary epithelial cells [78], they have not been reported in breast cancer. Expression of the IGF-I receptor is correlated with good prognosis in hormone-dependent breast cancer [79]. Basic and acidic FGF, as well as FGF-5, are produced by normal mammary stromal fibroblasts, as is IGF-I [78,80]. Normal mammary epithelial cells are stimulated by these growth factors. Stroma of breast cancer patients produces IGF-II [81]. Breast cancer cell lines also have been shown to produce mRNA for all members of the FGF family, as well as IGF-related activities and IGF binding proteins [81–83] and platelet-derived growth factor (PDGF) [75]. IGF-, PDGF-, and FGF-related growth factors may contribute to stromal–epithelial communication in tumors. This communication could be bidirectional, with growth factors such as TGF-α, PDGF, and FGF originating in the epithelium and stimulating the stroma. in turn, the stroma could secrete factors such as IGF, FGF, and TGF-α-related factors to support early, or even later, malignant lesions. Perhaps another major function of FGFs and other factors released by breast cancer is in promoting tumor blood vessel infiltration, a process known as angiogenesis [90].

During development of the mammary gland, roles have been suggested for some of these tyrosine kinase-related growth factors [35]. EGF, TGF-α, and their receptors have been previously described in both the ductal growth of puberty and in the lobuloalveolar growth of pregnancy/lactation. IGF-1 is important predominantly in the ductal growth, while insulin is particularly important in lobuloalveolar growth. Insulin, IGF-I, and IGF-II are all capable of strong mitogenic effects on human breast cancer as well [81–84]. Members of the FGF family are less well studied in their effects on mammary development. However, both basic FGF (bFGF) and acidic FGF (aFGF) can stimulate normal human mammary epithelial cells in culture [78,85]. In mouse mammary epithelial cells in culture, bFGF was shown to be both growth stimulatory and dedifferentiating (casein synthesis was inhibited) [86]. Interestingly, it has been shown that histologically normal human mammary epithelial cells are not as responsive to bFGF as breast-carcinoma-derived cells [85]. Additionally it has been shown that, in contrast to breast cancer adenocarcinoma cell lines, normal mammary epithelial cells and the milk derived, and immortalized HBL-100, all produce bFGF. Among breast cancer cell lines, carcinosarcoma Hs5789 was shown to produce bFGF [87].

The receptors for the insulin, PDGF, and FGF families are all tyrosine kinases but have different structures. FGF and PDGF receptors are similar to EGF receptor but have a split tyrosine kinase domain. Both represent an expanding family of receptors. [88,89] Insulin and IGF-I receptors are tetrameric proteins, with two disulfide-linked extracellular ligand-binding α subunits and two intracellular tyrosine-kinase-containing β subunits. It is not yet known which growth factor receptors are of primary importance in

kinase. Recognition of the receptor by coated pits results in internalization into endosomes and distinction in lysosomes [71].

It is not clear how genetic alterations during the process of breast tumorigenesis might interact with the EGF receptor signal transduction mechanism. It is possible that the receptor function could change as a result of modulation of receptor turnover kinetics, coupling of kinase to a particular substrate(s), or more distal modulation of a substrate function to alter gene control.

## 5. Other tyrosine kinase receptor–growth factor systems in breast cancer

Most of the cell surface receptors that appear to be important in positive regulation of mammary epithelial cells possess a tyrosine kinase activity. As previously described, the EGF receptor is probably the best understood. A second member of the EGF receptor family, c-*erb*B-2 also appears to be quite important, particularly in the context of its overexpression in malignancy [72,73] The structure of c-*erb*B-2 is quite similar to that of the EGF receptor. An external domain, transmembrane domain, and cytoplasmic domain with tyrosine kinase region are all present. Signal transduction through the receptor appears to be possible in a manner quite similar to that of the EGF receptor. This has been particularly well documented in several studies demonstrating that in fibroblasts, hybrid receptors with the EGF extracellular domain fused to the c-*erb*B-2 transmembrane and cytoplasmic domains can respond to EGF with production of a growth signal. (see Chapter 11). A new family member, c-*erb*B-3 has also been recently identified in breast cancer [74]. However, the implications of c-*erb*B-3 for breast cancer biology or prognosis are unknown at present.

Until recently, no ligands for c-*erb*B-2 had been identified. A 30-kDa TGF-α-related species has been isolated from the conditioned medium of the hormone-independent MDA-MB-231 breast cancer cell line (and identified in some other hormone-dependent and -independent breast cancer cell lines [7,26,50,75–77]). When tested on cells containing EGF receptor, such as fibroblasts, normal mammary epithelial cells, and hormone-dependent breast cancer cells, the purified growth factor is stimulatory. However, on cells expressing high levles of c-*erb*B-2, in addition to the EGF receptor, the growth factor derived from MDA-MB-231 cells is inhibitory [50]. It is not yet clear if inhibition can be obtained in vivo or if 30-kDa growth factors from other breast cancer cell lines have this characteristic. The TGF-α-like molecule from MDA-MB-231 cells is capable of displacing the monoclonal antibody 4D5 from its epitope on c-*erb*B-2 and appears to be the first candidate ligand for c-*erb*B-2 receptor. The exact relationship between the gene for this protein and TGF-α remains to be determined since sequencing has not been reported.

will probably not be of primary importance in autocrine growth regulation of malignant and metastatic disease. For example, though hormone-dependent MGF-7 cells both synthesize TGF-α and respond to its exogenous addition in vitro and in vivo, no autocrine significance can be strongly suggested [61]. In addition, the CAMA-1 cell line responds to estrogen but not EGF; it does not possess EGF receptors [62]. Also, TGF-α or EGF receptor gene transfection into MCF-7 or ZR 75-1 cells will not allow a significant growth advantage for the cells in vitro and in vivo [63,64]. It seems likely that the EGF receptor-TGF-α system may be much more critical in normal gland growth and early stages of breast tumorigenesis. In spite of this, therapeutic strategies employing EGF receptor ligands or antibodies coupled to toxins or therapeutic drugs could conceivably find future therapeutic utility, since a large portion of hormone-independent breast cancers express significant levels of this receptor, even though a direct function in most breast cancer tumors of the receptor has not been proven [39,65,68].

## 4. EGF receptor function and signal transduction

The EGF receptor serves to regulate the proliferation of multiple tissues in fetal development, nonreproductive adult life, and pregnancy [67]. The ligand-receptor system appears to be quite ancient because all seven of the ligands described to date are structurally related to *Drosophila notch*, *delta*, and *slit* genes and to nematode *lin-12* and *glp-1* genes [68]. The growth factors of this family are usually synthesized from transmembrane precursors and are processed by proteolytic cleavage to yield the soluble final form. Though such processing usually occurs, it has been recently shown that the uncleavaged precursor can act on the receptor of an adjacent cell in a mode of action termed *juxtacrine*. The characteristic three disulfide linkages in the secondary structure of all members of this growth factor family are all required for growth factor action [69].

The EGF receptor is a transmembrane protein with extensive external glycoslylation. Signal transduction is thought to require growth factor binding and receptor dimerization within the plane of the membrane. Signal transduction is mediated through the c-*src* oncogene-like kinase domain, while kinase substrate specificity involves recognition by an additional cytoplasmic domain amino terminal to the kinase. Several receptor substrates of interest for signal transduction studies have been identified: phospholipase Cγ, phosphoinositol-3γ kinase, GAP, MAP kinase, raf kinase, and several growth factor receptors. Receptor function appears to be attenuated *via* protein kinase C-mediated phosphorylation of a submembranous threonine residue [70]. In addition, receptors are internalized through some mechanism involving yet another short cytoplasmic region amino terminal to the

the early proliferation of breast cancer vs. later stages in tumor progression to more malignant forms.

## 6. The *myc*, *fos*, and *jun* nuclear oncogenes in breast cancer: Interaction with estrogen and growth factors

The nuclear protooncogenes — c-*myc*, c-*fos*, and c-*jun* — appear to be of particular interest in human breast cancer. c-*myc*, c-*fos*, and c-*jun* are all nuclear protooncogenes. c-*fos* and c-*jun* form heterodimeric complexes called AP-1 and regulate multiple genes through a defined consensus sequence [91]. Based on recent studies, c-*myc* also appears to form a heterodimeric complex with another protein called *max* (or *myn* in the murine species) and to modulate a different set of genes through a different consensus sequence [92]. In many systems, c-*myc* acts to promote cell proliferation, and to inhibit differentiation. On a molecular level, proliferation appears modulated by c-*myc* as a result of its regulation of initiation of DNA replication. The carboxyl end of the c-*myc* protein appears to be essential for cell transformation and for autosuppression, the most clearly defined gene regulation activity of the protein [93].

c-*myc* protooncogene can confer immortality to fibroblasts [94] and alter fibroblast responsiveness to growth factors [95,95]. In human primary breast cancer, c-*myc* amplification and overexpression have been reported in 15–40% of tumors [97–100]. c-*myc* amplification has not yet proven to be associated with clinical staging or other known prognostic variables, and has been found to correlate with poor prognosis in only one study so far [100]. Recent reports have suggested that c-*myc* expression may alter resistance to cis-platinum and other DNA strand-scission inducing drugs [101] and may suppress differentiation in association with the suppression of collagen gene transcription [102]. c-*myc* is also able to suppress transcription of the rat *neu* oncogene (c-*erb*B-2 homologue in murine species) expressed in an NIH 3T3 cell line [103]. The relevance of this observation in mammary tissue or for the human c-*erb*B-2 homologue is not yet known, but the study emphasizes a potential role of c-*myc* as a transcription regulator. c-*myc* amplification also does not seem to be associated with c-*erb*B-2 amplification in primary breast cancer [104]. It is also of interest that mutationally activated c-*myc* and v-*myc* genes can suppress endogenous c-*myc* transcriptional initiation when transfected into rat-1 fibroblasts [105]. c-*myc* and N-*myc* have been recently shown to bind the retinoblastoma gene product [106].

In vitro studies have also introduced the *myc* gene into immortalized human or mouse mammary epithelial cells using an amphotropic retroviral vector. In immortalized mammary epithelial cells transfected either with *myc* or SV40T nuclear oncogene (but not v-*ras*$^{H}$ or v-*mos*), it was observed that the cells could be stimulated to grow in soft agar by bFGF, aFGF,

EGF, or TGF-α [78,107]. Since normal human diploid human fibroblasts produce EGF- and FGF-related growth factors [78,80] and their conditioned media can support transforming growth of nuclear oncogene transfected mammary epithelial cells, these observations may have relevance in vivo for stromal-epithelial interactions. These data suggest that c-*myc* might function in early breast cancer lesions to allow growth factors or hormones to act to drive aberrant, transformed growth.

The nuclear protooncogenes are also induced when human breast cancer cell lines are stimulated to proliferate in monolayer culture in vitro by estrogen and progesterone [108–111]. Estrogen induces c-*fos* and c-*jun* within one-half hour and c-*myc* within 1 hour of treatment. It is not yet clear whether these nuclear protooncogenes are necessary or sufficient for estrogen action. However, a recent study has addressed c-*myc* regulation during tamoxifen therapy of breast cancer patients. This study demonstrated that tamoxifen treatment of estrogen-receptor-positive breast tumors resulted in a significant decrease in c-*myc* mRNA [112]. Conceivably, estrogen and progesterone may modulate cell proliferation through simultaneous modulation of growth factor and receptor synthesis, and through the induction of nuclear protooncogenes to sensitize the tyrosine kinase-mediated growth controls.

c-*myc* protooncogene may have a special relationship to breast cancer in older women. Increased amplification of c-*myc* has been observed in human breast cancer tissue in postmenopausal patients. It is possible that this reflects cumulative proliferation and/or contributes to aberrant mitogenic responses in postmenopausal breast cancer [97]. c-*myc* expression is also enhanced in many other tissues as aging progresses [113].

## 7. Growth factors, their receptors, and tumor prognosis

The potential roles of TGF-α or EGF in transformation may also involve alterations in the expression and function of their receptor, the EGF receptor. Clinical evidence for an association of increased expression of the EGF receptor and its structurally related homolog, c-*erb*B-2 with more aggressive, hormone-unresponsive breast cancer has accumulated in recent years [65,66,72,73]. This is also supported by studies in vitro of cultured primary human breast cancer biopsies [114] and in established human breast cancer cell lines [39].

EGF receptor (c-*erb*B) is a protooncogene that has been frequently found to be overexpressed in human breast cancer tumors compared with normal breast tissue. Approximately 35–50% of human primary and metastatic breast tumors possess, to varying degrees, EGF receptors as measured by dinding of $^{125}$I-EGF to isolated membrane fractions [114]. Increased levels of EGF receptor expression that are observed in primary breast tumors or in breast cancer cell lines are not generally due to gene amplification, but

rather are due to an increased level of EGF receptor mRNA and protein expression [115]. However, amplification of the EGF receptor has been detected in MDA-MB 468 cells, a human breast cancer cell line that possesses approximately $2-3 \times 10^6$ EGF binding sites/cell [116]. Several clinical studies have suggested that EGF receptor status may be an important independent prognostic factor in human breast cancer [117–121]. High levels of EGF receptor expression are associated with tumors that have higher proliferative rates and in patients with axillary lymph node involvement and early cancer recurrence [118–124]. In axillary lymph-node-positive breast tumors, EGF receptor expression appears second only to lymph node involvement as a prognostic marker in terms of both relapse-free and overall survival [119,125]. Moreover, in a study in which multivariate analysis was performed, only EGF receptor expression among estrogen receptor status, tumor size, and histologic grade has been found to be predictive for disease-free and overall survival in breast cancer patients with axillary lymph-node-negative disease [125].

Several studies have also demonstrated that there is a statistically significant inverse relationship between the presence of estrogen receptor content and the levels of EGF receptor expression in human primary breast cancer [117,118,122,123,125]. In this respect, overexpression of EGF receptors in breast tumors of elderly patients treated with primary endocrine therapy has been associated with a poor response to endocrine treatment [126]. In addition, EGF receptor status has also been correlated with the lack of response to endocrine therapy in patients with breast cancer relapse after surgical treatment [127]. An inverse correlation between estrogen receptor expression and EGF receptor expression has also been observed in several human breast cancer cell lines [128]. However, it is unclear if there is any causal relationship between high levels of EGF receptor and the acquisition of an estrogen-dependent phenotype. This is emphasized by one study that failed to convert estrogen-receptor-positive ZR-75-1 cells to an estrogen-receptor-negative phenotype with EGF receptor transfections [63].

TGF-α mRNA expression has been found in 40–70% of primary human breast tumors [129–131]. In one study, in situ hybridization demonstrated that TGF-α mRNA was expressed in breast tumor cells and not in surrounding stromal cells or in any infiltrating host lymphoid elements [130]. No significant correlation could be found between the expression of TGF-α mRNA and steroid receptor status, axillary lymph node involvement, or patient relapse [129,130]. In a small series of human breast cancers, the majority of tumors (59%) were found to coexpress TGF-α and EGF receptor mRNAs, suggesting that a potential autocrine loop may be operative in vivo [131]. The enhanced level of expression of TGF-α mRNA that is observed in human breast tumors is not due to any alterations in the TGF-α gene, since no evidence of rearrangements or gross amplifications of the TGF-α gene could be detected in these tumors [130]. Immunoreactive and bioactive TGF-α has been found in 30–50% of primary human breast

tumors, of which 50% of the TGF-α positive tumors had TGF-α levels that exceeded the levels of growth factor detected in benign breast lesions or in normal mammary tissues [132–134]. In one study, a significantly higher immunoreactive-TGF-α content could be detected in tumors that were estrogen receptor and progesterone receptor positive [134]. However, other studies have failed to find such an association or to have higher levels of TGF-α in estrogen and progesterone receptor negative tumors [126,130]. TGF-α protein has also been found approximatelly 50% of the lymph node metastases [133]. Tamoxifen treatment of breast cancer patients resulted in a tenfold reduction in the TGF-α expression in the tumor [134]. Studies have not yet clearly determined whether EGF receptor or TGF-α overexpression is correlated with c-*myc* overexpression or whether their coexpression relates to tumor prognosis.

Since the first report that demonstrated c-*erb*B-2 protooncogene amplification in approximately 25% of 189 human primary breast cancers [3], a large number of different studies have been published on the role of c-*erb*B-2 oncogene amplification and overexpression in breast cancer [3, 72,73]. The c-*erb*B-2 protooncogene amplification and/or overexpression of its p185 protein product has been detected in 10–30% of primary human breast tumors and in several human breast cancer cell lines. Several clinical studies have also shown that p185 c-*erb*B-2 protein overexpression is frequently associated with a more aggressive disease, since it is a significant predictor of reduced overall survival and the time to relapse in women with breast cancer [3,135]. Moreover, c-*erb*B-2 gene amplification and/or overexpression of p185 c-*erb*B-2 has been positively correlated with axillary lymph node involvement, poor nuclear grade, hematogenous metastases, estrogen-receptor-negative tumors, aneuploid cancers, and a high rate of proliferation [3,73,135]. Overexpression of c-*erb*B-2 and EGF receptor in the same tumor is associated with a poorer prognosis than overexpression of either oncogene alone [136]. More controversial is the role of c-*erb*B-2 protooncogene status as a prognostic indicator in node-negative breast cancers [3,73]. c-*erb*B-2 does not appear to be associated with c-*myc* amplification, and no studies have suggested a particularly negative impact in association with their coamplification.

Activation of c-*neu*, which is the rat c-*erb*B-2 gene, occurs through a single point mutation. The single point mutation occurs in the sequence encoding the transmembrane region that converts a valine residue to glutamine, glutamic acid, or aspartic acid. However, no activating transmembrane mutations have been detected in the c-*erb*B-2 protooncogene in primary human breast tumors. This suggests that overexpression of the normal p185 *erb*B-2 protein is involved in human breast tumors [72].

Some experiments have been carried with cell lines in vitro to address the relevance of c-*erb*B-2 amplification. Overexpression of the normal rat c-*neu*, or overexpression of the human c-*erb*B-2 genes using a strong retroviral promoter, led to transformation in vitro and in vivo of mouse NIH-

3T3 fibroblasts. In addition, it has recently demonstrated that overexpression of the c-*neu* protooncogene in the immortalized human mammary epithelial MCF-10A cell is able to induce a transformed phenotype in vitro [137].

Several other protooncogenes have been analyzed for gene amplification and/or overexpression in human breast tumors. *int*-2 and *hst*-1 are two FGF-related protooncogenes located on chromosome 11q13. Coamplification of these two genes has been detected in approximately 9–15% of primary human breast tumors and appears bo be associated with a poor prognosis [3]. However, protein overexpression has not yet been shown to be associated with these amplifications.

## 8. In vivo models of breast cancer: Transgenic mice

Recently, transgenic mice have been established that overexpress in the mammary glands c-*myc*, v-Ha-*ras*, and activated c-*neu* (c-*erb*B-2) oncogenes, alone or in combination, to assess their transforming potential in vivo. The results may enhance our understanding of the human disease. Two different groups have succeeded in overexpressing the c-*myc* protooncogene in mammary tissue using either the MMTV long terminal repeat (LTR) [138] or the whey acidic protein (WAP) gene promoters [139] to drive expression. The WAP promoter expresses exclusively during lactation, while the MMTV LTR driven by glucocorticoid or progesterone will cause expression much earlier in pregnancy and in a wider range of tissues, including salivary glands, male accessory glands, and several secretory glands of the head. Multiparous females of 2 of 13 separate founder MMTV-*myc* transgenic lines developed moderately well-differentiated mammary adenocarcinomas. A much higher frequency (80%) of mammary tumors was observed in 3 of 3 founder lines of WAP-*myc* transgenic mice, in which mammary tumors were observed 2–3 months after the initial onset of lactation. The mammary tumors in WAP-*myc* mice appeared to be well differentiated, acquiring constitutive expression and secretion of β-casein, as well as transgenic *myc*. These studies clearly establish the possibility that *myc* is a potent mammary tumorigenic agent. However, the requirement for initial overexpression to occur at pregnancy-lactation and the emergence of extraordinarily well-differentiated tumors may represent significant deviations from human breast cancer. The effects of transgenic *myc* overexpression on virgin mammary tissue and the characteristics of any resulting tumors have not been described.

A novel ex vivo–in vivo approach using *myc* expression directed by a retroviral vector has also been utilized in mice [140]. Edwards and coworkers infected primary cultures of mouse mammary epithelial cells with *myc* and then retransplanted them back to cleared mammary fat pads. This technique allows regrowth of the mammary network and facilitates the detection of abnormalities in growth or differentiation caused by the oncogene. These

studies demonstrated the c-*myc* infectants grew as a hyperplastic ductal network with ducts more densely packed than normal. The impact of this hyperplastic growth on the development of cancer was not evaluated.

Another ex vivo–in vivo approach [141] has utilized the chicken virus-derived v-*myc* expressed by retroviral vector in chicken embryo fibroblasts (CEF). The fibroblast growth in vitro in anchorage-independent soft agar growth was enhanced by EGF. In addition, the v-*myc*-CEF cells were implanted in the choriallatic membrane of the chicken embryo to assess growth effects n vivo. Coimplantation of v-*myc*-CEF with irradiated rat-1 cells secreting recombinant EGF led to the formation of vascular proliferation and invasive tumors. Neither v-*myc*-CEF nor irradiated rat 1-EGF cells alone led to tumors, and the study demonstrated that v-*myc* sensitizes cells in vivo to paracrine tumorigenic effects of the MMTV LTR [142,143] or the WAP gene promoter [144].

Other studies have used the membrane-associated v-$ras^H$ oncogene for comparison to *myc*. MMTV-*ras* transgenic mice developed mammary tumors with very high frequency, even in virgin mice. Sinn and coworkers reported that 10 of 13 different founder lines developed multifocal, stochastic mammary adenocarcinomas, appearing as early as 1 month. Tremblay and coworkers generated similar mice, in which 4 of 4 founder lines exhibited mammary tumors between 4 and 10 months. MMTV-*ras*-specific mammary lesions appeared in both males and females, and malignancies of salivary, lung, and lymphoid tissue were observed as well. The WAP-*ras* construct was significantly less potent, inducing mammary tumors in less than 1% of transgenic mice with a latency of approximately 1 year [144]. The reason for the difference in potency of the MMTV-*ras* and WAP-*ras* transgenes is not clear but probably represents differences in the activities of the promoters themselves.

Sinn and coworkers also produced double transgenic animals by mating MMTV-*myc* and MMTV-*ras* mice [145]. The two oncogenes were found to act synergistically in male and female mammary tissue, with a tumor incidence at a rate that was more than the additive rate of either alone. However, even the expression of both oncogenes in the same mammary gland was not sufficient to cause complete malignant transformation, as monoclonal mammary tumors appeared stochastically. In this case, mammary tumor latency was not significantly different when comparing WAP-*myc* mice and the double-oncogene WAP transgenic animals. This result is perhaps not so surprising, considering the effects of WAP-*ras* alone on mammary tissue and again probably reflects specific properties of the WAP promoter.

Two laboratories have used the same general strategy to study the consequences of overexpressing activated c-*erb*B-2 in vivo [146,147]. The MMTV LTR was used by both groups to drive expression in the mammary gland; however, their conclusions were markedly different. Muller and coworkers noted nearly a 100% incidence of polyclonal tumors arising

with extremely short latency in one line of mice in which activated c-*erb*B-2 is expressed uniformly throughout the mammary epithelium. This study strongly suggested that overexpression of activated c-*erb*B-2 was sufficient to transform mouse mammary tissue in vivo. In contrast, Bouchard and coworkers [148] reported that only independent monoclonal adenocarcinomas occurred stochastically in the female mammary gland in 4 of 4 founder lines between 5 and 10 months of age. The authors argue that activated c-*erb*B-2 was necessary but not sufficient for neoplastic transformation of mammary epithelium. The reasons for the differences in these two studies are not obvious; however, both studies clearly implicate the activated c-*erb*B-2 gene in the development of murine breast cancer. Implications of these studies for human breast cancer are unclear, since mutational activation of this oncogene is virtually unknown in women.

Transgenic mice have also been used to determine the effects of TGF-α expression on mammary gland development and tumorigenesis. These transgenic studies complement those using *myc*, *ras*, and *erb*B-2 protooncogenes in that they test the effects of an agent thought to function primarily in a proliferation (and/or differentiation) fashion. Hypothetically, *myc*, *ras*, and *erb*B-2 protooncogene activation and/or overexpression could by thought of as representing tumor initiation events, while TGF-α overexpression could represent a tumor promotional event. It has been proposed that increased cell proliferation (as induced by a tmor promoter) may function to increase cancer in the presence of background mutational events or carcinogenic initiators. In one study MMTV-TGF-α transgenic mice were generated, exhibiting terminal duct and alveolar hyperplasia in both virgin and pregnant animals in 3 of 10 founder lines. Lobular hyperplasia, cystic hyperplasia, adenoma, and adenocarcinoma (with long latency) were also observed [148]. Two other studies used the mouse metallothioneine promoter to drive TGF-α expression [149,150]. In both studies mammary adenomas and adenocarcinomas developed in multiparous transgenic females after a fairly long latency (6–14 months). Jhappan and coworkers [54] also reported a twofold increase in epithelial cell proliferation during glandular growth and increased ductular branching. Furthermore, delayed epithelial penetration into the fat pad was observed in pubescent TGF-α transgenic females, similar to earlier experiments in which EGF capsules were implanted near growing mammary epithelium [151].

Another hormone of mammary development, growth hormone (GH), has also been used to probe gland biology in the transgenic mouse system. Using the ubiquitously expressed HMGCOA promoter, overexpression of the human growth hormone gene resulted in precocious mammary gland growth and development, and milk-protein synthesis. Furthermore, postlactational gland regression did not occur — glandular differentiation persisted abnormally [152]. Mammary tumors were not noted, and it is possible that such premature differentiation observed with growth hormone will eventually be shown to have some impact on the tumorigenic process.

## 9. Summary

In summary, evidence is beginning to accumulate in support of a major role for tyrosine kinase receptors (and their activating growth factors) and steroid hormones and their receptors in normal development and differentiation of the mammary gland. A point of intersection of their mechanisms of action in growth control appears to be the induction of nuclear protooncogenes such as c-*myc*. When c-*myc* is amplified, as it is in many breast cancers, EGF and FGF receptor tyrosine kinase action becomes transforming, not simply mitogenic. A source of the transforming factors could be either stromal or epithelial [43,60,78,153,141]. This mechanism could function early in the progression of breast cancer. c-*erb*B-2 and EGF receptor overexpression and amplification, when they occur, appear to render tumors even more malignant and of especially poor prognosis. These mechanisms could function late in the progression of breast cancer. Transgenic mouse studies have begun to echo these themes. They have established that a growth factor (TGF-α) and its receptor (EGF receptor), which appear to be important in normal mouse and human proliferation and gland development, and a protooncogene (c-*myc*), commonly amplified and overexpressed in human and mouse breast cancer, can each contribute to mammary carcinogenesis. The mechanisms of the two are likely to be distinct. *myc* is likely to be acting as a tumor initiator in combination with normal proliferative factors, whereas TGF-α is likely to be acting as a hyperproliferative (promotional) factor in combination with a normal background of mutational events. The role of unmutated but amplified *erb*B-2 in the transgenic mouse is not yet known.

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PART IV

# Steroid hormone receptors

# 14. Characterization and regulation of estrogen and progesterone receptors in breast cancer

Linnea D. Read and Benita S. Katzenellenbogen

## 1. Introduction

The growth and proliferation of reproductive tissues are dependent upon the effects of a variety of modulatory substances, including the sex steroid hormones estrogen and progesterone, and an increasingly long list of growth factors. Like their nonmalignant counterparts, the growth, proliferation, and metastatic potential of breast cancer cells are markedly influenced by these substances.

Both in vivo and in vitro work in breast cancer tissue has shown that estrogen dramatically escalates proliferative and metastatic activity in these cells, in part via the induction of growth factors, proteases, and basement membrane receptors [reviewed in 1]. In contrast, progestin has been shown to have marked growth-inhibitory effects on these tumor cells [2–4] and is, in fact, often loosely referred to as an estrogen antagonist because of its ability to modulate estrogen action [5].

Current endocrine therapy for breast cancer includes the use of sex steroid hormone analogs. The antiestrogen, tamoxifen, has been used most widely as a first-line adjuvant therapy in postmenopausal women due to its minimal side effects [6,7]; antiestrogens have both cytostatic and cytotoxic effects on the growth of cells, and are so named due to their ability to suppress cell responses to estrogen. High-dose progestins [8], estrogen synthesis inhibitors [9], and most recently, the antiprogestin RU38,486 [10] have been used with similar degrees of success in ongoing clinical trials to halt or reverse tumor progresssion.

Both estrogen and progesterone are believed to exert their effects largely through interaction with nuclear receptor proteins. Histocytochemical localization [11], enucleation studies [12], and crosslinking analysis [13] indicate that the unoccupied form of these receptors is localized in the nucleus. Upon hormone binding, the receptor ligand complexes become tightly associated with chromatin and influence transcription [14,15]. Although receptors can be bound to hormone agonists or antagonists, receptors bound to antagonists fail to activate gene transcription or do so only poorly [16–19]. It is becoming increasingly clear that estrogen and

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

progesterone subtly influence both their own and each other's receptors, as well as other secondary messenger systems in cells.

Utilizing the present knowledge of the mechanism of hormone action, breast cancer patient candidates for hormonal therapy are chosen on the basis of their hormone receptor status. Approximately two thirds of tumors in breast cancer patients are positive for estrogen receptor (ER) (that is, contain high levels of the protein), and up to two thirds of these patients respond to antiestrogen therapy [20]; better response rates are seen in patients who are also positive for progesterone receptor (PR). Still, there remains one third of ER-positive patients who, for unknown reasons, do not respond to hormone therapy. Clinical trials [21–23] and in vitro growth assays [24] with concurrent antiestrogen/progestin treatment have not shown a greater effect on growth inhibition than either therapy given alone; in fact, these treatments are only more successful when the drugs are given alternatingly [21–23]. It is clearly of great importance to understand more fully how the effects of these hormones relate to steroid receptor function and dynamics.

A decade ago it was possible to study the dynamics of ER or PR only at the protein level, using hormone binding assays and crude sedimentation profiles to characterize these proteins. More recently, monoclonal antibodies [25–29] against these proteins and cDNA clones for these receptors [30,31] have been produced. With the recent burst of molecular biology techniques, it has become possible to more fully characterize the structure of the receptor proteins, as well as to study the properties, regulation, and dynamics of messenger RNA (mRNA) transcription, translation, and turnover.

For the last two decades, our laboratory has been interested in the mechanisms by which these substances, in particular estrogen and progesterone, modulate breast cancer cell behavior. In this chapter we will describe the characterization of the estrogen receptor protein as well as work on the regulation of estrogen and progesterone receptors.

## 2. Characterization of estrogen receptor

Early studies on the structure of estrogen receptor revealed it to be a protein of molecular weight 66,000 by SDS-polyacrylamide gel analysis (SDS-PAGE) [32], with a Stokes radius of 4.4 and a sedimentation coefficient of 4.1S [33–35]. In early studies, cell homogenization resulted in leakage of ER from the nucleus to the soluble cell fraction, making the ER appear to be cytosolic in location in its unbound state. However, with the production of monoclonal antibodies to ER [25,26], immunocytohistochemical localization, cell enucleation, and crosslinking studies made it clear that ER was located in the nucleus in both its unoccupied and occupied forms [11–13,36].

More information about the structure of ER itself came when its primary amino acid sequence was determined in 1986 [37,38]. Human ER is a protein of 595 amino acids. Through analysis of the amino acid sequence of ER, ER has been shown to be a member of a family of zinc-containing nuclear receptors that interact with DNA to modulate transcription of specific genes. Members of this family, which includes receptors for progesterone, glucocorticoid, thyroid hormone, vitamin D, and retinoic acid, contain two major regions of homology [37–40]. These regions, through deletion mutation analysis [39,40] and peptide mapping described below, have been identified as a DNA-binding domain [39,40] located approximately in the center of the receptor and a hormone binding domain (HBD) [39,40] in the carboxy-terminal region of the receptor.

Recently, two covalently attaching ligands for the ER have become available: tamoxifen aziridine (TAZ [41,42]), which acts biologically as an antiestrogen, and ketonestrol aziridine (KNA) [43,44], which acts as an estrogen agonist. The aziridine side chains on these molecules allow them to become covalently linked to the ER once they have bound it, labeling the ER with high efficiency and selectivity. These compounds allowed us to subject the bound receptor to rigorous assays, such as SDS-PAGE or high-pressure liquid chromotography (HPLC) without the risk of ligand dissociation during purification [45]. Using ER labeled with these affinity labels or detected with monoclonal antibodies to ER, structural analysis of the receptor using proteolytic digests helped define the hormone-binding and immunoreactive domains of the protein [32]. Digestion of covalently labeled ER with trypsin, chymotrypsin, or *Staphylococcus aureus* V8 protease found the TAZ labeling, as well as the epitopes for two anti-ER antibodies, H222 and D75, to be limited to a 6-kDa receptor fragment identified by SDS-PAGE analyses. This 6-kDa fragment is contained in the C-terminal portion of the protein in the hormone-binding domain (HBD); the HBD, previously identified by deletion mutation analysis, includes amino acid residues 302–553 [39]. Deletion mutational analysis localized the H222 epitope to a region within residues 462–528 [40].

As mentioned above, antiestrogens antagonize the actions of estrogens by competing with estrogens for binding to ER, but they fail to effectively activate gene transcription [16,17], in part by failing to recruit transcription factors necessary for the proper response [46,47]. Studies have also shown that ER bound to estrogens have different physiochemical characteristics when bound to antiestrogens [16,18,34,36]. It became of interest to elucidate the differences between the structure of ER when bound to either of the two classes of compounds.

To further describe the binding characteristics of the HBD, ER was radiolabeled with the antiestrogen $^{3}$H-TAZ or the agonist $^{3}$H-KNA, and was subjected to rigorous digestion with the proteases named above and also with cyanogen bromide. The resulting fragments were purified by HPLC and radiosequenced, and it was found that both the estrogen and

antiestrogenic compounds labeled the same residue, cysteine 530, found within the HBD near its carboxyl terminus [48].

The fact that both the estrogenic and antiestrogenic affinity labeling agents react covalently with the same cysteine indicates that differences in receptor–agonist and receptor–antagonist complexes do not result in differential covalent labeling of amino acid residues in the hormone–binding domain. The finding that these two agents label the same amino acid is consistent with the mutual competition of both TAZ and KNA with estradiol ($E_2$) for binding to receptor, and also probably reflects the high preference of these aziridine affinity labels for reaction with cysteine. Thus, when both of these agents are bound in the hormone-binding site of the receptor, cysteine 530 is the favored site of reaction for the aziridine function.

Confirmation of the importance of this amino acid residue came from experiments in which it has been observed that receptors containing amino acids 121–538 display $E_2$ binding of appropriate affinity, whereas more truncated receptors containing amino acids 121–507 show no $E_2$ binding at all, indicating residues between 507 and 538 are critical for hormone binding [49].

There are four cysteines in the hormone-binding domain (C381, C417, C447, and C530). Using recombinant DNA techniques, we have recently examined the importance of these four sites in the ER HBD [50,51]. Mutant human ERs were produced by changing each one of the four cysteines to alanine, and C530 was also mutated to serine. The wild-type and mutant receptors were then expressed in ER-negative Chinese hamster ovary (CHO) cells using an RSV-promoter-containing vector. These transfected receptors were then assayed for hormone binding and for their ability to activate estrogen-responsive reporter plasmids. All the mutants bound estradiol with a $K_d$ similar to wild-type receptor. They all also were capable of covalent labeling by TAZ and KNA, indicating, in the case of the C530 mutant, that the affinity label is attaching to another Cys residue in the HBD; this was confirmed with treatment of ER with low concentrations of methyl methanethiosulfonate, a cysteine-specific reagent, which reduces TAZ/KNA binding while not affecting $E_2$ binding [50,51].

Interestingly, however, although $E_2$-stimulated transcription was similar to wild type in the C381A (i.e., $Cys^{381} \rightarrow Ala^{381}$), C417A, C530A, and C530S mutants, the C447A mutant required 50x higher $E_2$ concentrations to achieve half-maximal transactivation. Additionally, the effectiveness of TAZ in inhibiting $E_2$-stimulated transcription and the ability of KNA to stimulate transcription was altered in the C530A and C530S mutants, but not in the other mutants tested [50]. That elimination of the cysteine and, hence, the possibility of covalent attachment at C530, reduced the effectiveness of TAZ as an antiestrogen suggests that covalent attachment at C530 is important in its antiestrogenic potency. However, since TAZ still does become covalently attached to the C530A ER, it indicates that in the absence of cysteine at position 530 an alternate residue, presumably

a different cysteine in the HBD, became labeled. Identification of this residue, currently underway, will provide further insight into the three-dimensional conformation of the HBD and the hormone binding pocket in particular. In addition, the impaired ability of estradiol to activate transcription in the C447A mutant highlights the importance of this region in ER function.

## 3. Regulation of estrogen receptor

### *3.1. ER half-life and mRNA content*

As mentioned in the introduction, estrogen, via the estrogen receptor, has potent influences on the proliferation and metastatic potential of breast cancer cells. Estrogen also stimulates the production of a variety of proteins; in MCF-7 breast cancer cells, $E_2$ induces plasminogen activator, pS2, progesterone receptor, cathepsin D, and other secreted proteins [52–55]. At the same time characterization studies of the structure of ER were being performed, investigators were interested in the dynamics of ER in cells and in what way it was modulated by hormones, growth factors, and other conditions. The availability of new techniques, and the production of monoclonal antibodies [25,26] and cDNA probes for ER [37,38] made it more possible than ever to follow the appearance and disappearance of ER with various treatments.

In most of the studies listed below, we and other investigators have made use of the cell lines MCF-7 and T47D, two breast carcinoma cell culture lines derived from pleural effusions of patients with metastatic breast carcinoma [56]. The MCF-7 cell line contains high levels of ER and estrogen-inducible progesterone receptor [57–60]. In contrast, the T47D cell line contains very low levels of ER but expresses PR at high constitutive levels [56,61–63]. Much of the information on the regulation of breast cancer cell function by estrogenic hormones is derived from studies of these cell lines.

After ER was first described, investigators used protein synthesis inhibitors to study ER turnover and effects of ligands; however, these studies were limited because the inhibitors would halt not only ER synthesis but also the machinery of the entire cell. In fact, we observed that cyclohexamide completely blocked ER turnover in uterine cells in primary cell culture [64]. In 1984, we performed pulse chase experiments with the covalently attaching antiestrogenic ligand tamoxifen aziridine (TAZ) [42] to measure ER turnover. We have also used density shift techniques employing the incorporation of dense amino acids into receptor [65–67], thus determining the half-life of newly synthesized protein. Using these techniques, we and others have found a receptor half-life in MCF-7 cell of approximately 4 hours. We obtained a similar half-life for ER in uterine cells in vitro, as well

as in intact uteri in rats in vivo, indicating that ER is indeed a rapidly turning over protein [64].

As described below, estrogen markedly decreases ER levels in tissues with high basal ER content. Much of the cell culture work done before 1986 was done using a pH indicator for cell culture media called phenol red. At that time we found that contaminants in phenol red preparations contained significant estrogenic activity [58,68]. Recent work on isolated nucleoplasts from MCF-7 cells suggest that ER protein half-life may be slightly longer, approximately 7 hours, in phenol-red-free conditions [69].

We and others have determined some of the hormonal factors and growth conditions (see Section 3.2 below) that influence the regulation of ER mRNA and protein levels in MCF-7 and T47D cell lines.

As several groups have shown [30,37,38], the ER mRNA appears as a prominant 6.6-kb band on Northern blot analysis when probed with the λOR8 cDNA probe. The message is expressed in high levels in MCF-7 cells; T47D cells contain approximately 25% of the ER mRNA content of that found in MCF-7 cells, and ER mRNA is undetectable in the ER-negative cell line MDA-MB-231, reflecting the relative content of ER protein contained in these three cell lines [70].

### *3.2. ER regulation by estrogens*

In MCF-7 cells, treatment with 17-β-estradiol ($E_2$) causes a dose-dependent reduction ($1/2_{max}$ effect at $10^{-11}$ M) of ER mRNA levels to 40% of control levels in 48 hours (Figure 1), mirroring the effect on protein levels as detected by ligand binding and immunoblot analysis [70].

The reduction of ER levels in MCF-7 cells by estradiol is completely blocked by the concomitant addition of 100-fold excess LY117018, a potent antiestrogen. When the cells are treated with antiestrogen alone, there is no effect on ER levels (Figure 1). The results of $E_2$ and antiestrogen treatments in these cells correspond well with dense amino acid labeling studies with MCF-7 cells in which the rapid ER turnover was slightly accelerated with estrogen exposure but was not influenced by antiestrogen exposure. In these earlier studies it was found that when receptor was bound with antiestrogens (CI628, nafoxidine, or the covalently attaching TAZ), there was no significant change in estrogen receptor turnover in MCF-7 cells [57,60,71]. In contrast, the half-life of ER bound to $E_2$ was only slightly faster (~3 hours as compared to 4 hours).

Reduction of receptor levels by agonist is a well-characterized event. $E_2$ has been shown to reduce ER protein in MCF-7 cells [57], and also in the ER-positive breast cancer cell line ZR-75 [72] and in other target tissues, such as rat uterus, chick oviduct, and endometrial cancer [73,74]. Additionally, many other hormones downregulate their own receptor: In cells containing high receptor levels, glucocorticoids, progestins (see below),

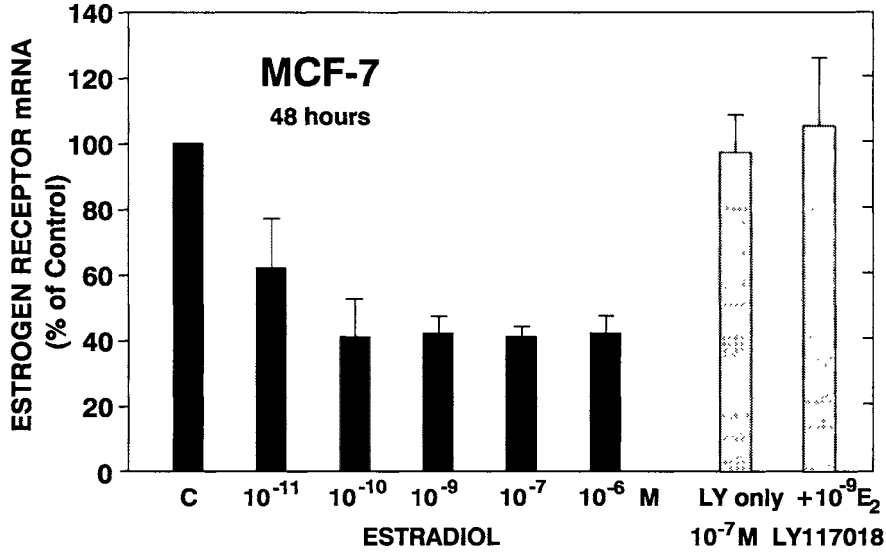


*Figure 1.* Dose dependence of estrogen receptor mRNA downregulation by $E_2$ and effect of the antiestrogen LY117018 in MCF-7 cells. MCF-7 cells were exposed to hormone or vehicle (0.1% ethanol) for 48 hours. Hormone and media were renewed daily. Messenger RNA was isolated by guanidine thiocyanate lysis and cesium chloride gradients. Poly(A) RNA was isolated by batch chromatography and quantified by Northern blot analysis. Bars represent the mean ± SD of three or four values from separate experiments. (Reprinted from Read et al. [70], with permission.)

vitamin D, and thyroid hormone have been shown to decrease their own receptor levels [62,63,75–81]. When this phenomenon was first recognized, it was called 'processing' [60] and was thought to be necessary for proper transduction of the hormonally controlled event. In studies in MCF-7 cells, ER 'processing' was greatest with treatment with $E_2$, partial with the antiestrogen tamoxifen, and did not occur with various other antiestrogens [57,60,71]. It is presently recognized that 'processing' is not necessary for many of the actions of hormones. Although processing is known to accompany $E_2$ action in MCF-7 cells [57,60], studies with the ER-positive cell line MDA-MB-134 have shown that $E_2$-induced growth is not accompanied by a loss of ER [82]. Additionally, in MCF-7 cells grown in low-estrogen conditions for many years, $E_2$ retains the ability to induce progesterone receptor without a concomitant loss of ER [70].

In contrast to findings with strongly ER-positive cell types, when cells containing low levels of ER are treated with estrogens ER levels rise. In T47D breast cancer cells, we observed that $E_2$ caused a rapid 2.5-fold increase in ER mRNA levels [70]. Similarly, in rat or *Xenopus* liver cells, and in liver tumor HepG2 cells in which untreated cells contain very low levels of ER, $E_2$ markedly increases ER levels [83–86].

### *3.3. ER regulation by progestins*

Progestin has been known for many years to antagonize the action of estrogen on the proliferation and growth of both normal and cancerous target tissues [52,87]. At the DNA level, the receptors for steroid hormones have been shown to compete for factors that mediate their enhancer functions [88]. Progestin has also been shown to induce estrogen metabolism in some cells [89]. We and others have also found direct effects of progestin on ER protein and mRNA levels.

In MCF-7 cells, $E_2$-induced decreases in ER can be reversed partially with the addition of the progestin R5020. The addition of the antiprogestin RU486 to these cells has no effect on $E_2$-dependent reduction of ER mRNA in these cells [70]. Interestingly, as we will see below, estrogen and progestin also have opposite effects on progesterone receptor (PR) mRNA and protein levels in these cells [62]. It should be noted that in MCF-7 cells PR is increased by $E_2$ and other factors (see below), but is undetectable in untreated cells. Therefore, studies in MCF-7 cells with progestins are somewhat constrained by the fact that the presence of PR is wholly dependent on estrogen priming, so progestin treatments must be given concurrently with $E_2$.

In T47D cells, which contain progesterone receptor protein levels approximately ten times higher than in our $E_2$-primed MCF-7 cells [62], the progestin R5020 has a very dramatic effect on downregulating ER mRNA and protein. ER message levels are reduced to 20% of control levels within 2 days of R5020 exposure; whereas, as in MCF-7 cells, the antiprogestin RU486 has no effect on ER mRNA levels. A reduction in ER protein by progesterone has also been shown previously in rat uterus [90] and in hamster uterine and uterine decidual cells [73,74,90–93]. The progestin ORG2058 has also been shown by Alexander et al. to reduce ER levels in T47D cells, and this drop is not associated with a change in ER mRNA half-life [94], suggesting that progestins may affect ER via a reduction in the ER mRNA synthesis rate. In the same study, indirect evidence linked the downregulation of ER by progestins in T47D cells with the ability of estrogen to activate an estrogen-responsive element, demonstrating the physiological relevance of measured reductions in ER protein and mRNA. As for the ER protein, studies employing dense amino acid incorporation-density shift analysis have revealed a shortened ER half-life in decidual cells treated with estrogen plus progesterone and have indicated that progesterone blocks estradiol-dependent ER protein synthesis in these cells [90,91].

### *3.4. ER regulation by growth factors*

MCF-7 cells, like many other hormone-responsive tissues, are known to produce receptors for a variety of growth factors and growth-inhibitory factors, and as is widely reported [95–100], our MCF-7 cells respond to

the growth factors epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) by increasing cell proliferation. The same cells respond to transforming growth factor beta (TGF-β) with a reduced rate of cell proliferation. Since $E_2$ is also known to increase cell proliferation in these cells, we were interested in investigating whether these factors altered ER levels, thereby affecting the sensitivity of these cells to estrogen. Using concentrations of the three factors that we found to modulate cell proliferation rates, we found that EGF and IGF-I elicited no change in ER levels over a 2-day period, whereas TGF-β had only a modest effect on ER levels [70]. Therefore, growth factor modulated growth responses do not seem to be reflected in the modulation of ER levels.

## 4. Regulation of progesterone receptor

For many years progesterone receptor (PR) levels in target tissues has been known to be controlled by steroid hormones. Estrogens increase PR levels in rat [101], rabbit [102], hamster [103,104], and guinea pig uterus [105], as well as in human endometrial [106] and breast cell cultures. Similarly, progestins decrease PR levels in these same tissues [101–104,107,108]. Recent work described below indicates PR in breast cancer cells is also regulated by protein hormones and growth factors [109].

As we have seen above, progestins can have marked effects on ER mRNA and protein in hormonally responsive cells. Progestins have additionally important effects on growth factors and their receptors, steroid synthesis and metabolism, and differentiaion and proliferation of breast cancer cells [for review see 5]. The ability of progestins to blunt or eradicate the proliferative response of breast cancer cells to estrogen is the basis for high-dose progestin therapy currently used in the treatment of breast cancers [2,3,8]. Since progesterone's response has been thought to be primarily mediated through the nuclear progesterone receptor, we and others have been interested in how PR is modulated in hormonally responsive tissues.

### *4.1. PR protein and mRNA*

Like ER, the purification and characterization of PR has been facilitated by the production of monoclonal antibodies to PR [27–29] and the cloning of the receptor [31]. As with other receptors of the steroid hormone receptor superfamily, PR is a nuclear receptor [110,111] with DNA-binding and hormone-binding domains; however, PR from most species and target cells, as detected by immunoblot analysis, appears as two forms, A and B, with approximate molecular weights of 90,000 and 120,000, respectively. Both A and B form doublets on SDS-PAGE analysis on exposure to progestin agonists [75] due to phosphorylation events. In vitro transcriptional activa-

tion studies have shown that the A and B forms differ in their ability to stimulate MMTV and ovalbumin promoters [112]. Interestingly, both the A and B forms of the receptor are encoded by the same mRNA, with the A form translation beginning at an interior AUG translation start site [113].

Using PR cDNA probes made against human PR isolated from T47D cells [31], PR messenger RNA in estrogen-primed MCF-7 cells and untreated T47D cells appears as five species of 2.8, 3.5, 5.3, 5.8, and 11.4 kb. All five messages are of sufficient size to code for the 120,000 molecular weight PR protein. Additionally, in all treatments we have performed all five mRNAs decrease or increase together in response to different hormone treatments.

Similar size messages for PR have been found by other workers in T47D cells [114–117], and several forms are observed in chick oviduct using a chick oviduct PR cDNA probe [118]. Work in chick oviduct has shown that the multiple-sized forms of chicken PR mRNA represent forms of mRNA with either 3′ or 5′ truncations and/or variations in the polyadenylation of 3′ untranslated RNA [119]. Similar modifications also appear to account for the multiple message sizes in T47D cells [120].

### *4.2. PR downregulation by progestins*

Treatment of the T47D cells and MCF-7 cells with the progestin R5020 and the antiprogestin RU486 has revealed interesting information about the ways PR is regulated in these two breast cancer cell lines [62]. From studies in T47D, it has been seen that the synthetic progestins R5020 and ORG2058 markedly downregulate the level of PR [62,63,75]. Studies employing dense amino acid-density shift analyses in T47D cells indicate that this progestin-mediated decrease of the PR protein reflects a marked increase in the degradation rate of the PR as well as a marked decrease in the rate of PR synthesis [63]. Evidence that PR is regulated at least in part at the transcriptional level includes the fact that PR mRNA is reduced temporally before the protein. As seen in Figure 2, PR mRNA decreased rapidly in R5020-treated cells over the first 6 hours of treatment, whereas the protein level dropped more slowly. Similarly, the RU486-treated PR mRNA reached a minimum in 6 hours, whereas the RU486-treated protein started increasing again after approximately 14 hours. Similar results have been duplicated in other labs using the natural ligand progesterone [115]. Although the progestin and antiprogestin-treated PR mRNA and protein values followed one another closely for the first 6 hours of treatment in both cases, after 2 days of treatment, the effects of the two ligands diverged. Possibly the initial binding of the ligand, regardless of its ultimate bioactivity as a progestin or antiprogestin, initially downregulates the receptor message (and consequently the receptor itself).

When observing the PR protein levels in T47D cells by immunoblot analysis, while PR protein is markedly downregulated by progesterone,

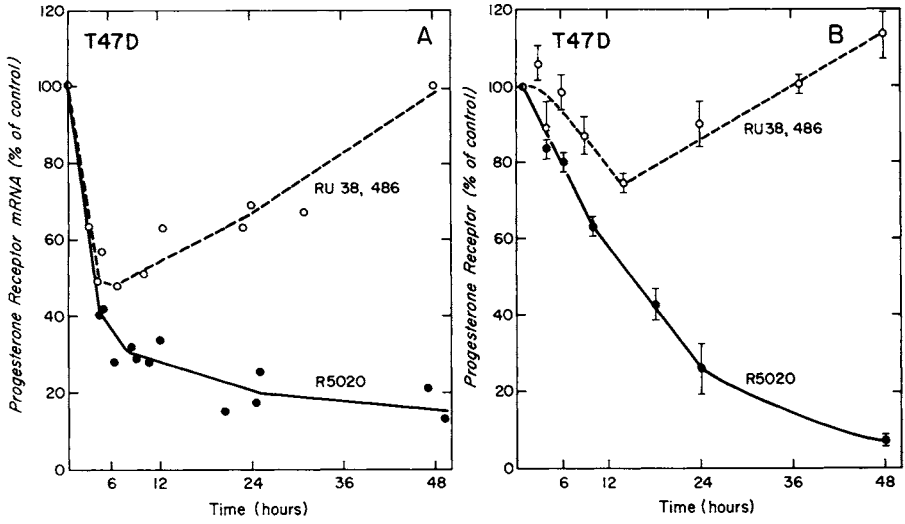


*Figure 2.* A, B: effects of R5020 and RU38,486 on progesterone receptor mRNA and protein levels in T47D cells. T47D cells were treated with 10 nM R5020, 10 nM RU486, or vehicle (0.1% ethanol) for 0–48 hours. Hormone and media were renewed daily. A: Effect on PR mRNA. Messenger RNA was isolated and quantified as in Figure 1. Values represent individual determinations from three experiments. B: Effect on PR protein. Cells were incubated with radiolabeled hormones, and then hormone binding was measured in salt-extracted nuclear receptor preparations. Bars represent the mean ± SE of three to six values. (Reprinted from Read et al. [62], with permission.)

R5020, and ORG2058 [62,115], the effects of RU486 are more transient and less pronounced. Recent studies using immunocytochemistry to localize PR in T47D cells have also demonstrated PR protein downregulation by R5020 and not RU486 [121]. In this regard, the effect of RU486 (which also acts as a glucocorticoid antagonist) is similar to that seen in IM-9 human lymphoblastoid cells in which RU486 evokes less processing of the glucocorticoid receptor compared with that evoked by dexamethasone, a synthetic glucocorticoid agonist [76].

It is presently thought that early events in PR:DNA interactions may be grossly similar in the presence of agonist or antagonists. When bound to either type of compound, PR binds to progestin-responsive element DNA [122,123] and has been shown to inhibit progestin-mediated transcriptional activation via competition at the progestin-responsive element [124] and impairment in its ability to recruit transcription factors [47]. RU486 is also thought to produce its effects as a progestin antagonist via some structural alteration of the receptor [122,125,126].

The effects of progestin and antiprogestin ligands on the PR mRNA and

protein in estrogen-induced MCF-7 cells are somewhat different from those observed in T47D cells. In both MCF-7 and T47D cells, progestin reduced PR mRNA and protein levels. However, in MCF-7 cells, the antiprogerstin RU486, as well as the progestin R5020, resulted in rather similar, ca. 50%, reductions in PR mRNA and protein levels [62]. Interestingly, since our T47D cells contain eight- to tenfold higher levels PR than are found in our estrogen-stimulated MCF-7 cells, the levels of PR remaining after R5020 exposure are quite similar in the two cell lines. It has been reported that R5020 reduces the half-life of PR protein in T47D cells from 21 to 6 hours [63]. In MCF-7 cells, R5020 reduces PR half-life, but the effect on receptor half-life from ca. 17 hours to 12 hours is not as marked [59]. Hence, the more striking effect of progestin of PR protein levels in T47D cells vs. MCF-7 cells is also reflected in a more marked reduction in PR mRNA.

### *4.3. PR regulation by estrogens*

It is well established that estrogen increases the PR content of MCF-7 cells and other target cells, such as those of the rat uterus [52] and brain [127]. Previous studies have shown that RNA and protein synthesis inhibitors prevent the estradiol-induced increase in PR protein in uterus [104,108]. Studies employing dense amino acids to study PR turnover in MCF-7 cells have also indicated that the effect of estrogen on increasing the level of PR protein is due solely to an increase in the rate of PR protein synthesis without alteration of the rate of PR degradation [128]. These observations are consistent with our findings that estrogen exposure markedly increases the PR mRNA content of MCF-7 cells [62].

In MCF-7 cells, estrogen causes a progressive increase in PR protein [128] and mRNA over several days of treatment (Figure 3). This stimulation is completely obliterated by the concomitant addition of the antiestrogen LY117018 [62], while antiestrogen alone has no effect on PR levels.

Since PR mRNA is virtually undetectable in MCF-7 cells in the absence of $E_2$, it follows that $E_2$ most likely increases PR at least in part by increasing PR mRNA synthesis. In MCF-7 human breast cancer cells, we find that $E_2$ causes a rapid, transient increase in PR synthesis within 3 hours as measured by nuclear runoff transcription analysis (NG and Katzenellenbogen, unpublished). An $E_2$-stimulated increase in the PR transcription rate in MCF-7 cells has been reported [129]. Whether estrogen-dependent stimulation of PR levels in breast cancer cells also reflects alteration of post-transcriptional events remains to be studied. In contrast to observations in human breast cancer cells in which $E_2$ appears to increase PR at least in part by altering the PR transcription rate, studies in chick oviduct reveal that $E_2$ does not increase PR transcription and that estrogen regulation of chick PR expression occurs solely at the post-transcriptional level [130].

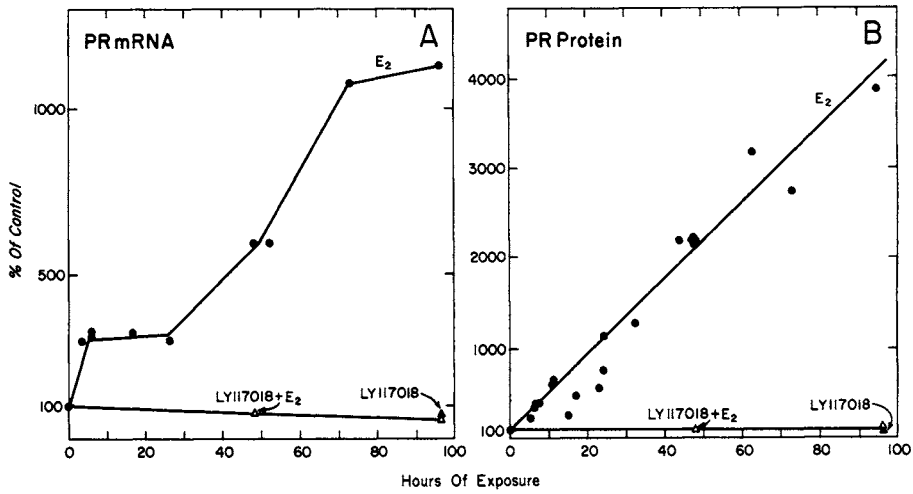


*Figure 3.* A, B: effect of $E_2$ and the antiestrogen LY117018 on the PR mRNA and protein content of MCF-7 cells. MCF-7 cells were exposed to 1 nM $E_2$ for 0–96 hours, to 1 nM $E_2$ plus 100 nM LY117018 for 48 or 96 hours, or to 100 nM LY117018 for 96 hours. Hormone and media were renewed daily. A: Effect on PR mRNA. Messenger RNA was isolated and quantified as in Figure 1. B: Effect on PR protein. Cells were harvested and assayed for PR protein by a whole-cell hormone binding assay. Values represent individual determinations from three to five separate experiments. (Reprinted from Read et al. [62], with permission.)

### *4.4. PR regulation by protein hormones, growth factors, and cyclic nucleotides*

We have found that in MCF-7 cells there is a marked serum dependence in $E_2$ stimulation of PR. $E_2$'s ability to stimulate PR (measured by ligand-binding cell assay or immunoblot detection with the antibody B39) increases as a function of percent charcoal-dextran-treated calf serum concentration, and at concentrations <1% serum $E_2$ has no effect on PR levels. However, serum given without $E_2$ at any concentration up to 10% is unable to induce PR levels, indicating that a permissive factor is present in serum, allowing $E_2$ to act on PR [109]. This observation was followed by a series of studies performed in our lab aimed at examining $E_2$ regulation of PR in MCF-7 cells in serum-free conditions. We attempted to culture cells in serum substitute media containing high levels of insulin. Suprisingly, we observed that PR levels became markedly elevated in these cells in the absence of estrogenic substances. The serum substitute was ITS+, containing insulin (6.25 μg/ml), transferrin (6.25 μg/ml), selenium 6.25 ng/ml, albumin (1.25 mg/ml), and linoleic acid (5.35 μg/ml). Interestingly, the ability of ITS+ to stimulate PR declined markedly as a function of serum concentration. Additionally, the ability of ITS+ and $E_2$ to stimulate PR was greater than additive at

intermediate serum concentrations (ranging from 0 to 10%). Analysis of the components of ITS+ revealed that insulin (6 μg/ml) alone was responsible for the stimulation of PR. This level of insulin is supraphysiologic; however, this concentration of insulin would be expected to allow it to bind insulin-like growth factor I (IGF-I) receptors. Further studies found that physiologic levels of IGF-I (10–40 ng/ml) were capable of evoking significant increases in PR.

The PR that was stimulated by IGF-I had similar affinity ($K_d$) to PR species induced by $E_2$ [109]. Additionally, the time course of increase of PR with IGF-I or ITS+ was the same as that previously found for $E_2$ [57,59,71,128], although the magnitude of the PR increase by IGF-I alone was slightly less than that evoked by $E_2$ or ITS+. More surprising, though, was the observation that the addition of excess antiestrogen (LY117018) suppressed the IGF-I-or ITS+-induced stimulation of PR. This suppression by antiestrogen could be reversed with $E_2$.

With respect to these observations, it should be noted that growth factors have been found to be important in the regulation of steroid hormone actions; among other examples, EGF and insulin have been shown to alter PR levels in guinea-pig fetal uterine cell cultures [131], insulin and IGF-I have been shown to be necessary for estrogen-dependent transcription in chick oviduct [132,133], and insulin and IGF-I are also needed for estrogen action on uterus and mammary tumors [134–136].

The findings that modulation of PR levels in breast cancer cells is under multihormonal/multifactor control led us to wonder how the pathway of action of estrogen, presumably acting directly at its nuclear receptor, might interact with the secondary messengers of IGF-I, a protein hormone acting at a membrane receptor.

Using primary cultures of immature rat uterine cells, we began to explore the signal-transduction pathways that might mediate multifactor regulation of PR [137]. The uterus, like breast cancer cells, has a PR induced by $E_2$ [52] and produces several growth factors and their receptors, including IGF-I, IGF-II and EGF [95,134,138–140]. As we found in the MCF-7 cells, IGF-I was capable of markedly increasing PR, and with the same time course as $E_2$. Again we found that the induction of PR by IGF-I was inhibited by increasing concentrations of charcoal-dextran treated serum. Additionally, we found that if we increased intracellular cAMP by treating the cells with cholera toxin plus isobutyl methyl xanthine (CT + IBMX) or by adding 8-bromo-cAMP, we were again able to induce PR seven- to eightfold (as measured by ligand binding or immunoblot analysis) just as with $E_2$ or IGF-I. In contrast, 8-bromo-cGMP did not induce PR over a wide range of concentrations [137]. Similar observations for stimulatory effects of cAMP on PR in fetal guinea-pig uterine cells have been made by Sumida and Pasqualini [141].

Interestingly, we found that we could suppress the cAMP-dependent PR induction with cyclic nucleotide-dependent protein kinase inhibitors (H8 or

PKI), and also that we could inhibit the action of cAMP with the addition of ICI164,384, a synthetic pure antiestrogen.

When given in combinations at individually maximally effective doses, we found that $E_2$, IGF-I, and CT + IBMX did not induce PR in a synergistic or additive fashion. In fact, using submaximal doses still did not permit PR levels to rise more than those obtained with the maximal concentration of one agent given alone [137].

Since the time course of action and the absolute levels of PR were similar in all these treatment groups, the question is raised whether they are acting through similar mechanisms [137]. This is being addressed in continuing studies, but the observation that antiestrogen and protein kinase inhibitors were able to not only suppress the action of cAMP, but also $E_2$ and IGF-I, suggests that there are some common components of the pathways, such as ER and phosphorylation events involved in the regulation of the PR by these agents. Hence, these studies identify cAMP, as well as estrogen and IGF-I, as important regulators of the level of PR in target cells and suggest that multiple factors, including those affecting intracellular cAMP levels, might influence responsiveness to progestins via regulation of the intracellular PR content.

## Acknowledgments

Support of the research from our laboratory described in this article from the National Institutes of Health, American Cancer Society, and Komen Foundation is gratefully acknowledged.

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# 15. Estrogen and progesterone receptor structure and action in breast cancer cells

Saverio Bettuzzi, Alan Robinson, Robin Fuchs-Young, and Geoffrey L. Greene

## 1. Introduction

The elucidation of the molecular mechanisms responsible for the hormonal control of cell proliferation in breast cancer has been the object of intense research. Because most breast cancers are initially dependent upon estrogens for continued growth, much of this research has focused on the role of estrogen receptor (ER) in the control of gene expression and mitosis [1], and on its use as a marker for hormone responsiveness and prognosis [2]. In addition, progesterone receptor (PR), as both a mediator of hormonal responses and as a product of estrogen action on breast cancer cells, has been studied extensively as a tumor marker [3] and in terms of its regulation by estrogen agonists and antagonists [4]. Although its function in breast cancer is unknown, the presence as well as the induction of PR has been coupled to estrogen-induced proliferative responses in breast cancer cells. An improved understanding of the function and regulation of expression of these transcription factors is emerging from studies of the structure, composition, and dynamic of the receptor proteins and the genes that encode them. The cloning and molecular analysis of all of the known steroid receptors has led to the definition of common functional domains and a proposed mechanism by which they interact with responsive genes, via *cis*-acting DNA enhancer elements, in normal and neoplastic tissues [5–7]. For ER and PR, these studies have been aided by the availability of a number of monoclonal antibody probes directed against specific regions of each receptor [8,9]. In addition, the same antibodies have been used to develop validated quantitative and histochemical immunoassays for ER and PR in a variety of hormone-responsive tissues and related cancers. Such assays have proved particularly useful in the evaluation of ER and PR in breast tumor extracts [10], in frozen and paraffin-embedded tissues and tumor sections [11–15], and in needle biopsies [16,17]. This paper summarizes the results of recent studies on ER and PR structure, composition, and activity in breast cancer cells as a function of agonist and antagonist binding.

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

## 2. Steroid receptors — General considerations

The estrogen and progesterone receptors, like all of the steroid receptors, are members of a large family of *trans*-activating transcription factors that are activated by a ligand and bind with high affinity and specificity to short DNA enhancer elements called hormone response elements (HREs). Interaction of steroid-receptor complexes with responsive genes in vivo can result in either up- or downregulation of transcription, depending upon the target gene and the tissue [5,6]. The molecular mechanisms by which either pathway occurs are still not known, although it is generally believed that for transcriptional activation, receptor-DNA complexes recruit, or allow the recruitment of, other transcription factors that comprise a functional transcription complex. This process might involve protein-protein interactions between receptor and other factors, resulting in the formation of DNA loops [18] to accommodate long stretches of DNA between promoters and HREs, or possibly by altering the local chromatin organization [19] to permit access of other transcription factors; obviously, both processes could occur. Transcriptional inhibition by steroid receptors may also involve more than one mechanism. Recent data suggest that in some systems (e.g., prolactin gene) [20] suppression may involve the interaction of receptor regions outside of the DNA-binding domain with promoter elements and tissue-specific factors, whereas in other systems (e.g., the osteocalcin gene) [21], the receptor may bind to specific HREs and sterically hinder the binding of a *trans*-activator. Similar results were observed for an ovalbumin-globin reporter gene (OV-GLOB), which was used to assess the effect of the *B* and *A* forms of chicken PR (cPR) on the transcriptional regulation of OV-GLOB in the presence of human ER (hER) [22]. The *B* form of cPR partially suppressed the hER-mediated induction of OV-GLOB in transfected chicken embryo fibroblasts, whereas the smaller *A* form of cPR actually enhanced the hER effect. However, for an MMTV-CAT reporter gene, cPR *B* and *A* were both stimulatory, although *B* was five times more efficient than *A*; hER had no effect. A third possibility is that receptor may interact directly, or indirectly, with other transcription factors (e.g., Jun) or steroid receptors to form heteromers that can have both positive and negative transcriptional activity [23]. In addition, one or more members of a heteromeric complex may interact with mixed DNA elements or half-sites in a responsive gene. It seems likely that control of transcriptional activity is a complex process that reflects the cooperative interaction of receptors, other specific and nonspecific transcription factors, various combinations of *cis*-DNA elements, and chromatin structure.

## 3. Human estrogen receptor

### *3.1. Structure and properties*

The isolation, sequencing, and expression of ER cDNA from MCF-7 human breast cancer cells has provided a wealth of information about the composition and organization of various functional domains in the estrogen receptor [24]. A comparison of amino acid sequences among all members of the steroid receptor family, coupled with functional analyses of in vitro generated mutants, has identified regions essential for at least four functions of steroid receptors, namely, ligand binding, nuclear localization/translocation, DNA binding, and transcriptional activation. The most highly conserved region is now known to be the 66 amino acid DNA-binding domain (Figure 1), and it is this region that has been used to define the members of a superfamily of regulatory proteins that includes the steroid receptors. This region can be further divided into two subregions of cysteine clusters tetrahedrally coordinated to zinc, analogous to the zinc 'fingers' found in the *Xenopus* transcription factor IIIA. The hydrophobic region in the carboxy-terminal portion of the ER molecule contains not only the ligand binding domain, but also a ligand-dependent transcription activating region, as well as a constitutive (hormone-independent) translocation signal [25]. In addition, it is probably this general region, by analogy to GR [26], that interacts with the hsp90 heat-shock protein in vitro, although this has not been demonstrated for ER. It is also this region that is responsible for

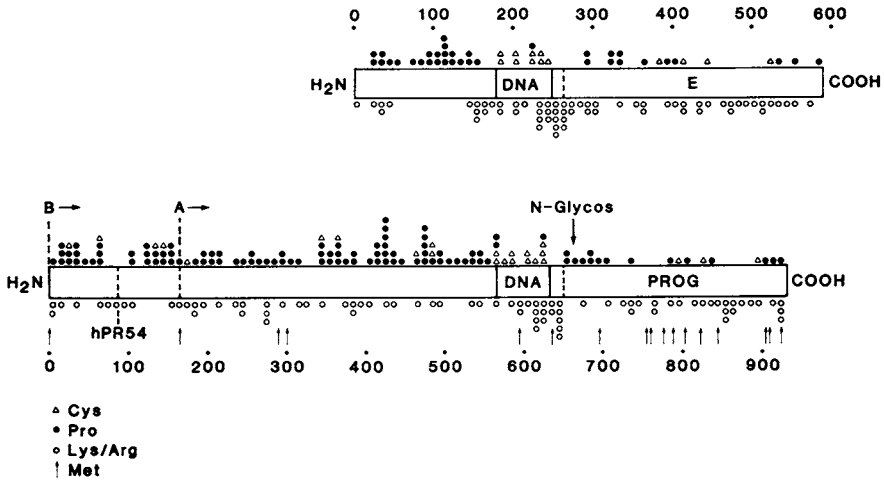


*Figure 1.* Schematic amino acid comparison between MCF-7 human estrogen receptor (upper) and T47D human progesterone receptor (lower). The two representations are aligned to make the 66-amino acid DNA-binding domains coincide. The putative initiation ATGs for the *B* and truncated *A* forms of hPR are shown.

the dimerization of ER [27]. The amino-terminal portion of ER apears to be required for maximal ER transcriptional activity and may contain more than one transcription activating region, one of which may be cell type specific [28]. Multiple transactivation domains may be a general feature of steroid receptors.

In regard to defining the structure and function of the hormone-binding domain of ER, we have succeeded in locating one possible contact point (cys 530) by covalently labeling human ER either with $^{3}$H-tamoxifen aziridine (an antagonist) or $^{3}$H-ketononestrol aziridine (an agonist) [29]. Following fragmentation of labeled ER with CNBr, trypsin, or V8, sequence analysis of purified peptides revealed that the site of attachment for both ligands had to be a cysteine at position 530, which is very close to the carboxy-terminal end of the defined hormone binding domain (position 538) [30]. We have therefore demonstrated that an estrogen agonist and antagonist can bind to the same site on the ER molecule, suggesting that these molecules regulate ER activity by differential alteration of the conformation of ER.

### *3.2. Interaction of hER with DNA*

As described above, the DNA binding domain of each steroid receptor appears to contain all of the information needed for a target-specific interaction with an appropriate HRE, although the nature of this interaction remains to be better defined. It has been suggested that the first finger motif is responsible for sequence specificity and that the second finger may stabilize protein-DNA interaction through nonspecific DNA binding [31]. Several recent studies [32] have more precisely localized specificity and DNA contact to the region between and immediately following the second pair of cysteines in the first finger. For estrogen receptor, the known response elements (EREs), such as those found in the chicken and *Xenopus* vitellogenin A2 genes, are palindromic sequences with 5-bp stems separated by a 3-bp spacer [33]. Single copies of these elements are able to confer significant estrogen inducibility to reporter genes containing heterologous promoters, such as the chloramphenicol acetyltransferase (CAT) gene fused to the thymidine kinase promoter, when transfected into ER-containing cells. A half-site ERE [34] that may involve the Fos/Jun complex [35] has been found in the chicken ovalbumin gene and more recently in the human PR gene [36,37]. By gel shift analysis, the specific, high-affinity interaction of purified MCF-7 ER (>90% pure) with the perfect palindromic vitellogenin A2 ERE contained in a 27-mer synthetic oligonucleotide was demonstrated [38]. Both halves of the ERE palindrome appear to be in contact with the receptor complex, which suggests the formation of a complex containing a head-to-head dimer of ER bound to the ERE, with each monomer recognizing one half of the palindrome. These results are consistent with extensive in vitro data that indicate the formation of a 5S homodimer of ER when receptor is activated. Purified ER has also been characterized by us as

an activated dimer. Interestingly, it was recently reported that binding of ER to ERE may require a 45-kDa single-stranded DNA-binding protein [39]. However, we have not observed a similar phenomenon with the purified human ER.

### *3.3. Expression of human ER in heterologous cells*

A major goal has been to express hER cDNA in various eukaryotic cells in order to study the properties and dynamics of human ER in homologous as well as heterologous systems, and to produce large quantities for structural studies. High-level expression ($3-6 \times 10^6$ molecules per cell) of functional full-length human ER was achieved by cadium selection of Chinese hamster ovary (CHO-k1) cells stably cotransfected with plasmids encoding MCF-7 hER and metallothionein [24,40]. The human ER isolated from these cells forms a classical 8–9S complex under hypotonic conditions, which suggests that associated nonsteroid-binding components (e.g., heat-shock protein, hsp90) are present in nontarget cells in sufficient quantity to complex ER that is 50–100 times more abundant than in MCF-7 cells. The human ER appears to be fully functional in CHO cells, even though it contains an artifactual mutation (gly → val) at residue 400 that results in tenfold lower affinity for estradiol at 25°C [41]. An unexpected but intriguing observation was the sensitivity of CHO-ER cells to estrogens. In cells expressing the highest levels of hER, estrogens were cytotoxic. The partial antagonist hydroxytamoxifen was equally toxic, whereas the complete antagonist ICI-164 was not. It is still not clear whether some form of ER-mediated squelching is occurring, or whether induction or suppression of a gene(s) involved in replication might be occurring. Studies designed to address this question are in progress.

Regardless of whether the unoccupied receptor is present in the cytoplasm or nucleus of a target cell, it is proposed to exist as a complex consisting of one steroid-binding protein, a dimer of hsp90, and possibly one or more small RNA molecules, as has been reported for unactivated rat glucocorticoid receptor [42]. A schematic representation of the possible composition of the different froms of ER observed in vitro is shown in Figure 2. Phosphorylation sites exist on both the hsp90 and receptor proteins. In CHO-ER cells, estrogens induce rapid increased levels of phosphorylation of ER (unpublished data). Also, preliminary data suggest that serine residues are involved; no evidence of tyrosine phosphorylation has been observed, although it has been reported that tyrosine phosphorylation of ER is required for steroid binding [43]. Recent experiments have shown a synergistic action of estradiol and cAMP on the induction of various ERE-tk-CAT reporter plasmids in Hela, CHO, and MCF-7 cells that express either recombinant or natural hER. This response has both hormone dependent and independent components. However, the presence of hER is absolutely required. What is not yet clear is whether hER is

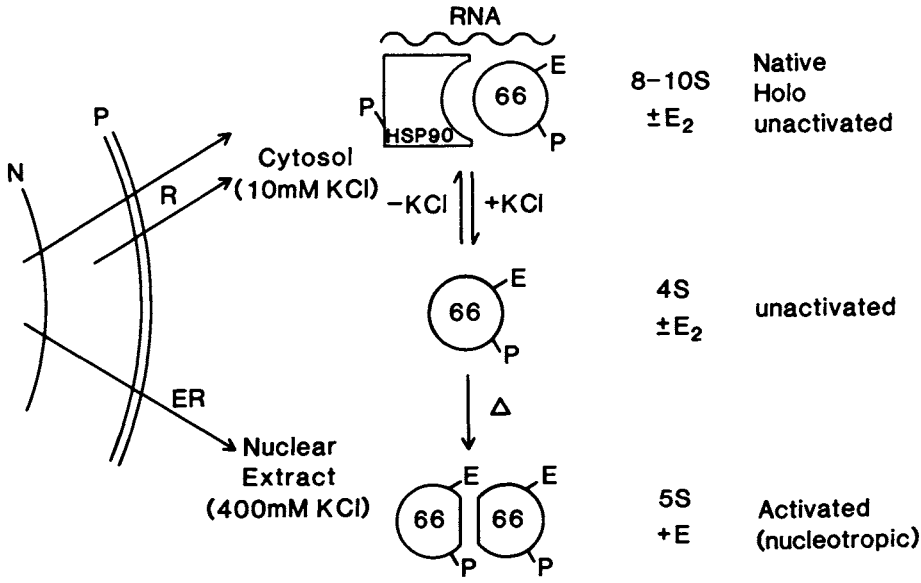


*Figure 2.* Model of various in vitro forms of estrogen receptor isolated from a hormone responsive cell. E = estrogen; $E_2$ = estradiol; P = phosphorylation site(s); Δ = heat. See text for details.

being directly phosphorylated in response to cAMP or whether hER is interacting with another protein whose activity is stimulated by cAMP. We are attempting to elucidate the mechanism of this phenomenon.

In regard to the subcellular location of ER in the absence of hormone, a wealth of data now support the idea that this receptor, and probably all other characterized members of this family except the glucocorticoid and mineralocorticoid receptors, are nuclear proteins [8]. Overexpressing CHO cells transfected with human ER cDNA show a nuclear localization of ER when stained with the H222 antibody by an indirect immunoperoxidase technique in cells that were grown in phenol red-free medium containing charcoal-stripped serum [44]. Little or no specific ER staining is observed in the cytoplasm of any of these cells unless the cell is undergoing mitosis. Thus, the translocation signal(s) encoded within the ER molecule does not appear to require hormone to be active, unlike the glucocorticoid receptor [45]. Recently, Picard et al. [25] demonstrated that the human ER contains only one constitutive nuclear localization signal, located in the hinge region (aa 256–303), whereas the glucocorticoid receptor contains a second signal in the hormone binding domain that is dominant and requires hormone. Apparently the progesterone receptor also contains two nuclear localization signals [46], and the one located in the hinge region is dominant and constitutive. However, unlike GR, the second signal in PR involves both the DNA and hormone-binding domains.

### *3.4. Regulation of ER expression*

The regulation of ER mRNA and protein levels in breast cancer cells is complex and apparently is dependent upon the hormonal history of the cells. In studies carried out in collaboration with Benita Katzenellenbogen [47], both up- (T47D) and down- (MCF-7) regulation of ER by estrogens were observed, although only MCF-7 cells that were maintained in normal calf serum displayed downregulation of ER. MCF-7 cells grown in charcoal-stripped serum were basically unaffected by short-term estrogen treatment. Differential regulation of ER by estrogen antagonists, progestins, and progestin antagonists was also observed, whereas several growth factors had only minimal effects on ER levels. Thus, it appears that the steroid hormones themselves are the dominant factors in ER regulation, at least in the breast cancer cell lines tested. Other laboratories have reported estrogen-mediated downregulation of ER in MCF-7 cells [48], and one of these groups [49] observed no regulation of ER in the particular T47D cell line used in their study. Progestin-mediated downregulation of ER mRNA in MCF-7 and T47D cells has also been reported [50]. A close correlation between protein and mRNA levels has been observed in all of these studies, consistent with transcriptional regulation, although post-transcriptional effects have been proposed [48].

## 4. Human progesterone receptor

### *4.1. Purification and immunochemical analysis*

The purification of human PR by steroid affinity chromatography and/or immunoadsorption, and the characterization of 14 rat and mouse monoclonal antibodies has been described [9]. PR from T47D human breast cancer cells consists of two steroid-binding forms (*A*: 88–93 kDa; *B*: 109–119 kDa); the origin of these forms remains controversial, as discussed below. Highly purified PR migrates as 93-kDa and 119-kDa progestin-binding proteins in SDS gels. In all, 13 monoclonal antibodies have been obtained that recognize epitopes shared by both forms of PR. One mouse immunoglobulin (KC146) is completely specific for the larger *B* form. The epitope for this antibody is present on all PRs tested, including the *B* form from chicken oviduct, whereas nine other antibodies recognize only human or nonhuman primate PR, and the remaining four crossreact with rabbit PR. Interestingly, two antibodies (KD67 and KD68) do not recognize PR in monkey oviduct and thus appear to be specific for human PR. This discrimination between a human and nonhuman primate steroid receptor has not been observed previously for any of the characterized receptor antibodies.

### *4.2. Cloning of human progesterone receptor cDNAs and chromosomal DNAs*

As part of our effort to understand the structural and molecular aspects of hPR gene regulation by several receptor–ligand complexes, genomic DNA and T47D cDNA clones encompassing the entire translated portion of hPR mRNA and approximately 7 kb of the 5′ untranslated sequence have been isolated, sequenced, and used to create CAT reporter plasmids as well as expression vectors for hPR isoforms. A comparison of corresponding human PR and rabbit PR sequences shows considerable homology in the 5′ untranslated portion of the two PR genes. In contrast, the corresponding region of the chicken PR mRNA (366 bp) is not homologous to either mammalian PR mRNA. The transcription start site for the full-length *B* form of T47D hPR has been reported [36] (Figure 2). A long 5′ untranslated sequence (743 bp) containing several small open reading frames and an in-frame stop codon precede the $AUG_B$ initiation codon for hPR (Figure 3). Like the rPR genomic sequence, the hPR genomic sequence upstream from the putative transcription start site contains several possible regulatory elements, including a CAACT sequence at position −98, which may correspond to a CAAT box. Both genes have a high GC content in this region and both have putative binding sites (TGGGCGGGCC) for the transcription factor Sp1.

When aligned with human ER *via* the DNA-binding domains, all of the additional PR sequence appears as an extension of the amino-terminal portion of the molecule (Figure 1). Like ER, the PR protein contains a high proportion of prolines in the amino-terminal half of the receptor, as well as a cluster of ten cysteines in the DNA-binding domain, nine of which are conserved with respect to ER, and a cluster of basic residues in and around the DNA-binding domain. The amino acid sequence homology between human ER and PR is about 56% in the 66-amino acid DNA binding domain and about 28% in the hormone-binding region. There is little homology between ER and PR 5′ to the DNA binding domain.

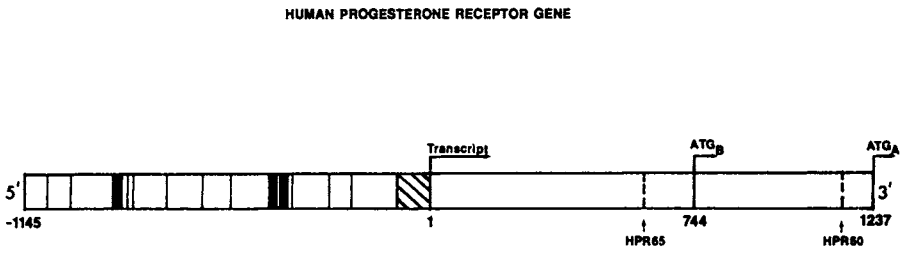


*Figure 3.* Schematic drawing showing the organization of genomic hPR DNA. Positions of the putative initiation site for transcription (n 1146) and the first two ATGs in the open reading frame are shown. $ATG_A$ begins at position +493 relative to $ATG_B$. Start sites for the HPR65 and HPR60 cDNAs are also indicated.

Because progesterone receptor expression can be induced by estrogens and variably suppressed by progestins in reproductive tissues and several breast cancer cell lines, it is of interest to determine the role of the corresponding receptor proteins in this regulation. Several studies suggest that both ER and PR may directly modulate the level of transcription of the human PR gene in MCF-7 and/or T47D cells. We therefore analyzed the 5′ untranslated hPR sequence for the presence of potential ER response elements (EREs) and PR response elements (PREs). Although reporter plasmids containing portions of this region have been shown to respond to ER in cotransfection experiments [37], well-defined ERE/PRE sites have not yet been identified. Interestingly, a 600-bp region (−2.3 to −1.7 kb) that occurs only in the human hPR gene contains several striking palindromic sequences, including a possible PRE. Recently, this putative PRE was shown to bind hPR selectively in vitro, suggesting that it may play a role in the autoregulation of hPR gene expression. Several other potential estrogen receptor (ER) and hPR binding sites, some of which coincide with similar sites in the rPR gene, were also found in the 3-kb region preceding the hPR promotor.

### *4.3. Regulation of PR expression*

In collaboration with Benita Katzenellenbogen [4], we have studied the effects of estrogens, progestins, and their antagonists on PR protein and mRNA levels in several breast cancer cell lines. By Northern blot analysis with human PR cDNA probes, PR mRNA appears as five species of 11.4, 5.8, 5.3, 3.5, and 2.8 kb; these species are absent in the PR-negative MB-231 and LY2 cell lines. In T47D cells, both the receptor and its mRNA levels are reduced by 90% within 48 hours of treatment within the synthetic progestins R5020 or ORG2058. In contrast, treatment with RU38,486, a progestin antagonist, reduces receptor and mRNA only transiently. In MCF-7 cells, PR mRNA and protein are virtually absent in the absence of estrogens. Treatment with estradiol induces both, in parallel, about 10- to 40-fold within 3 days. Antiestrogens (e.g., LY117018) block this effect completely. Interestingly, progestins and progestin antagonists both reduce receptor and mRNA levels in MCF-7 cells, although only by 40–60%. Clearly, the regulation of PR expression is different in the two cell lines. However, there is a close correlation between protein and mRNA levels, and the changes appear to be directly mediated by the ligands, presumably via their cognate receptors.

### *4.4. Expression of B and A forms of hPR*

An issue that still remains unresolved is the relationship, derivation, and functional differences of the two reported hormone-binding forms of mammalian and avian PR. Among the members of the steroid receptor

transcription factor family, only PR and possibly androgen receptor are reported to exist in two ligand-binding forms. Estimates of PR molecular weights way from 78 to 95 kDa for the smaller A form and from 108 to 120 kDa for the larger B form. Gene cloning data for chicken, rabbit, and human PR indicate the existence of only one gene for these PRs, although multiple forms of PR mRNA have been observed. The most likely explanations for the appearance of *A* in cell-free extracts are (1) that A is a proteolytic fragment of B, as suggested for rabbit and human PR [51], (2) that A is derived by translation of PR mRNA from an internal methionine initiation codon, as suggested for chicken PR [52,53], or (3) that there are two classes of mRNA, one containing $AUG_B$ and one containing $AUG_A$, as suggested recently for both chicken [54] and human [36] PR. The putative translation start sites for the *B* and *A* forms of T47D PR are identified in Figures 1 and 2. Although rabbit and human PR have been reported to occur exclusively as 110-kDa species in extracts or translation mixtures containing protease inhibitors, most studies show that mammalian and avian PRs exist in both forms under a variety of tested conditions. In fact, we have never observed the conversion of the T47D B form to A in any cell-free system. An alternative explanation is that the cleavage of B to A occurs in vivo via a specific proteolysis mechanism, although there are no data to support this hypothesis.

To determine whether the B and A forms of human PR are derived from the same mRNA via two independent translation initiation sites, full-length (hPR65) and N-terminal truncated (hPR60) expression vectors were created and tested in an in vitro translation system and in transiently transfected Cos-1 cells. When the hPR65 transcript was translated in vitro and the $^{35}S$-labeled products were analyzed by SDS-PAGE in 10% gels, the dominant $^{35}S$-labeled protein produced was the full-length *B* form (120 kDa) of hPR; less than 5% of the smaller *A* form was observed. When the hPR60 transcript was similarly translated, a major 90-kDa $^{35}S$-labeled protein was observed, along with a very weak band migrating just behind the 9-kDa protein. We have been unable to convert the full-length B protein produced in vitro to the 95-kDa *A* protein, even after prolonged incubation at 37°C in cytosol or nuclear extracts from T47D cells. In fact, both translated proteins were stable under all tested in vitro conditions. These data are consistent with the hypothesis that *A* cannot be generated from the mature B protein, at least not in vitro.

When hPR65 and hPR60 were expressed in Cos-1 cells, the 120-kDa and 90-kDa B and A forms of hPR were obtained in both cytosol and nuclear extracts of cells transiently transfected with the pSVL vectors. Western blot analysis with antibodies (JZB39 and KD68) that recognize both hPR forms revealed a dominant 120-kDa protein for hPR65 and a minor, but significant (ca. 5–20%) amount of immunoreactive 90-kDa protein, especially in the hypotonic cytosol extract. Therefore, the full-length transcript appears to be capable of producing the smaller A form of

hPR, presumably via initiation from $AUG_A$, even though B is dominant. For hPR60, the dominant immunoreactive species was a 90-kDa protein in both extracts. COS cells transfected with empty pSVL plasmid do not contain any hPR-immunoreactive protein in either extract. Although transfected cells were grown in charcoal-stripped serum to remove any endogenous progestins, a significant percentage of both immunoreactive hPR forms was found in the high-salt (400 mM KCl) 'nuclear' extract. When cells were treated with 10 nM $^3$H-ORG2058 for 30 minutes at 37°C, a portion of both forms of expressed hPR became more tightly associated with nuclear components, as judged by the disappearance of immunoreactive hPR from the low-salt extract. Interestingly, an upward shift in the migration rate of some of the hPR was observed for both forms after hormone treatment. These small changes in apparent molecular weight may represent increased levels of phosphorylation of the two proteins, as has been observed for progesterone receptor derived from various mammalian and avian tissues and breast cancer cell lines treated with progestins.

### *4.5. Transcriptional activity of the B and A forms of hPR*

To assess the relative ability of the *B* and *A* forms of hPR to regulate the transcriptional activity of a progestin-responsive gene, Cos-1 cells were cotransfected with an MMTV-CAT reporter plasmid (pMSG-CAT) and PR65 and/or hPR60. The MMTV long terminal repeat contains a hormone response element that is sensitive to both glucocorticoid receptor and progesterone receptor stimulation of transcriptional initiation. When comparable amounts of either form of hPR are expressed transiently in Cos-1 cells contransfected with MMTV-CAT, the B form of PR is four- to sixfold more effective than the A form of PR in stimulating hormone-dependent CAT expression. The progestin antagonist RU486 significantly inhibited the hormone-induced stimulation of CAT expression and had little or no effect on CAT activity when used alone. Thus, both expressed froms of hPR are capable of stimulating the transcription rate of a progestin-sensitive reporter gene, at least in heterologous Cos-1 cells. However, the full-length *B* isoform is significantly more efficient than the *A* form in regulating the PRE present in the MMTV long terminal repeat fused to CAT cDNA. These results are consistent with the observed preferential regulation of a similar MMTV-CAT reporter construct by the chicken PR *B* protein in cotransfected HeLa cells and in primary chicken embryo fibroblast (CHEF) cells [22]. Similar results were reported recently for human PR [36]. Thus, at least in transfected heterologous cells, the N-terminal domain of chicken and human PRs can specify target gene activation. What is not resolved by these studies is whether the *B* or *A* forms of PR preferentially regulate the expression of different target genes in the same cell under physiologic conditions. More information about the activities of other promoter elements

and/or additional tissue-specific factors will be required to better define the role of the N-terminal domain the differential activation of target genes.

Obviously, there are still a number of key dynamic and molecular aspects of receptor activity that are not resolved at this time. With the antibody, cDNA, and genomic DNA probes that are now available for ER and PR, it is now feasible to define both biochemical and genetic aspects of receptor activity in the coordinate regulation of gene expression in hormone-responsive tissues and cancers.

## 5. ER and PR immunoassays

The development of immunoassays for hER and hPR in hormone-responsive tissues and neoplasms has provided a wealth of information about receptor dynamics, such as nuclear localization in the absence of hormone, and about the existence and location of target cells for estrogens and progestins. We have continued to use our monoclonal antibodies to measure, characterize, and localize ER and PR in normal and neoplastic target tissues and their extracts from human, nonhuman primate, and rodent sources. Many of these studies are collaborative. Both quantitative and histochemical immunoassays continue to be evaluated extensively on breast cancer specimens throughout the world. Several studies [12,14,15,17] have shown that both ER- and PgR-ICA are predictors of endocrine response in patients with advanced mammary carcinoma and survival in women with Stage I or Stage II disease. Although studies such as these indicate that ICA analyses for ER and PR may be more informative than conventional ligand binding assays, the numbers are still relatively small and additional data are required to establish their clinical value. These methods have also proved useful for the evaluation of breast tumor needle biopsies [16], endometrial cancers [55], and ovarian cancers. A particularly promising application of ER and PR immunocytochemistry has been the mapping of ER- and PR-expressing cells in the brain and pituitary of guinea pigs [56], rats, and some species of monkey. Correlations of ER expression with neurological and endocrine signaling in these tissues will allow detailed assignment of control mechanisms in the central nervous system. Clearly, immunocytochemical analysis of PR in target tissues and cell lines is proving to be a powerful analytical tool for studying hormone responses.

## Acknowledgments

These studies were supported by Abbott Laboratories, the American Cancer Society (BC-86), and the NCI (CA-02897).

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# 16. Progesterone receptors in breast cancer

Candace A. Beck and Dean P. Edwards

## 1. Introduction

Steroid hormone receptors are members of a gene family of ligand-dependent transcriptional activators that also includes receptors for vitamin D, thyroid hormone, retinoic acid, and a number of transcriptional activators with as yet unknown ligands. Cloning of receptor genes and mutagenesis studies have revealed that steroid receptors are modular proteins organized into the functional domains shown in Figure 1. A highly conserved DNA binding domain (region C) located in the central portion of the molecule contains two zinc finger structures. The carboxyl terminal region (E) harbors domains for steroid binding and receptor dimerization. The amino terminus (A/B) is highly variable, both with respect to amino acid sequence and length, and is important for maximal transcriptional activity [1–4]. The progesterone receptor (PR) in breast cancer cells and in several normal reproductive tissues is produced as two steroid-binding proteins of different lengths, termed *PR-A* and *PR-B* [5,6]. The two proteins arise from a single gene either by initiation of translation at two different start sites within the same RNA transcript or from separate mRNAs produced by the use of alternate promoters [7–9]. PR-A is a truncated version of PR-B, missing 165 amino acids present in the N terminus of PR-B (Figure 1). The two PR forms, therefore, are identical in sequence in their hormone and DNA binding domains. In human breast cancer cells, PR-A and PR-B have apparent molecular weights of ≈94,000 and ≈120,000 respectively [6]. This property of the progesterone receptor is unique among the sex steroid and glucocorticoid receptors. Receptors for estrogen (ER), androgens (AR), and glucocorticoids (GR) are all synthesized as single-sized proteins. The functional role of PR-A and PR-B are not known. They have been reported to exhibit some differences in target gene specificities, suggesting that expression of two PR forms from the same gene may provide a mechanism to expand functional diversity [10].

Nuclear receptors mediate the diverse effects of steroid hormones on the growth and development of normal and malignant tissues by regulating transcription of a limited set of target genes. The molecular mechanisms by

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

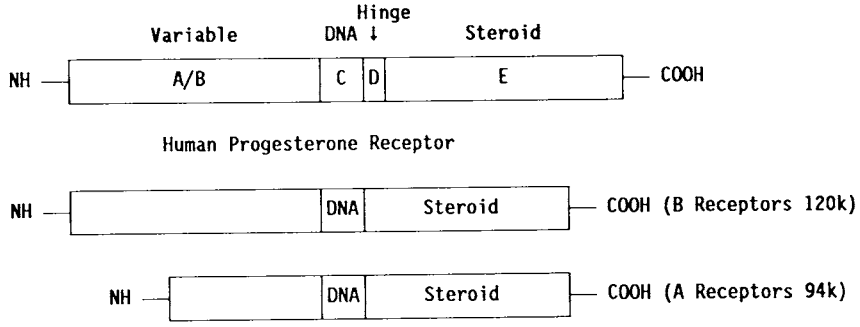



*Figure 1.* Structure of the human progesterone receptor.

which steroid receptors regulate target gene expression is an area of intense investigation. It appears that all nuclear receptors are inactive in the absence of hormone, requiring a hormone-dependent activation step in order to gain the ability to regulate gene transcription. The molecular mechanisms responsible for the process of steroid receptor 'activation' are not well understood. Steroid receptors also have the common feature of binding to specific *cis*-acting DNA sequences of hormonally regulated genes. These sequences, termed *hormone response elements* (HREs), have been shown by mutagenesis studies to be necessary for the induction of gene transcription by steroid hormones, implying that receptor binding to HREs is an important step in the regulation of target gene transcription [11]. However, binding to HREs appears not to be sufficient for receptor action, since receptors complexed with hormone antagonists are capable of binding to HREs but fail to affect transcription [12–15]. How receptor binding to HREs, in turn, affects gene transcription is not known. This likely involves complex patterns of protein-protein interaction between receptors and other transcription factors.

The hormone primarily responsible for stimulating cell proliferation in breast cancer is estrogen [16]. However, it is becoming increasingly apparent that progesterone also plays an important and perhaps as yet unappreciated role in the biology of breast cancer. In experimental breast cancer systems, PR have been shown to be functionally active in mediating transcriptional regulation of both endogenous progesterone target genes, such as fatty acid synthetase [17,18] and EGF receptors [19], as well as exogenous reporter genes introduced into breast cancer cells by gene transfection [12,13,20]. Progestins have been observed to be antiproliferative [21,22], behaving similar to the antiestrogen tamoxifen by inducing cells to accumulate in G1 of the cell cycle [23]. However, there are instances of experimental breast cancers in which progestins enhance growth [24,25] and stimulate the expression of growth factors and growth factor receptors [26]. High-dose progestin therapy is receiving renewed interest for the treatment of advanced breast cancer, either as a first-line or second-line endocrine

therapy. This renewed interest is due to low toxicities associated with high-dose progestins and to an efficacy similar to that of tamoxifen [27].

Much is to be learned about the role of progesterone in the biology of breast cancer. Toward this end it is important that we gain a better understanding of the fundamental properties of the progesterone receptor molecule. This chapter reviews our current understanding of the structural properties and basic molecular mechanisms of action of the progesterone receptor. Discussions will focus on studies of the human progesterone receptor in breast cancer cells. Analogies with PR in other experimental systems and with other members of the steroid receptor family have been included where appropriate. Studies from our laboratory have also been included to illustrate points of discussion.

Most of our studies have been conducted with the PR-rich T47D human breast cancer cell line [28], utilizing the progesterone response elements (PRE) of mouse mammary tumor virus (MMTV) as a target progesterone responsive gene [13,20]. Study of the progesterone receptor has been greatly facilitated by the production and availability of receptor-specific monoclonal antibodies (MAbs). Several laboratories have produced MAbs to PR including those raised against PR purified from rabbit uterus [29], chicken oviduct [30], human uterus [31], and breast cancer cells [32,33]. Our studies have utilized two MAbs produced against PR isolated from T47D cells. One MAb, designated AB-52, is reactive with both the A and B forms of human PR, and the other, B-30, recognizes the B form of human receptors only [32].

## 2. Progesterone receptors form specific complexes with heat-shock proteins

Purification of unactivated progesterone receptor under native conditions has revealed that it is a heteromeric complex composed of the steroid-binding polypeptide physically associated with other cellular proteins. The unactivated receptor complex binds weakly to DNA/chromatin in nuclei of hormone-deficient cells and is recovered largely in the soluble cytosol fraction of cell lysates as an 8–10S species. Several laboratories have identified the major associated protein in the 8–10S unactivated complex as heat-shock protein 90 (hsp 90) [34–36]. This has been demonstrated for all the sex steroid receptors and for glucocorticoid receptors (GR) in a variety of target tissues and cells [37–40]. In addition to hsp 90, several laboratories have found that unactivated PR and GR contain other associated proteins, including heat-shock protein 70 (hsp 70) and a protein(s) in the size range of 55–59 kDa that has been initially identified to also be a heat-shock protein (hsp 56) [41–46]. Smaller molecular weight proteins of 54, 50, and 23 kDa, of unknown identity, have also been detected in the unactivated PR complex isolated from chick oviduct [47]. Estimates of the relative abundance of associated proteins in the unactivated receptor complex have

been made. Since hsp 90 is found in excess of receptor protein and is known to form stable dimers when free in solution, it has been suggested that the unactivated complex contains two molecules of hsp 90 per receptor molecule [48]. The other associated proteins appear to be present in a lower relative abundance than hsp 90, suggesting they are present in the ratio of 1:1 with receptor protein [41–46]. This estimated composition and stoichometry of associated proteins is consistent with molecular mass estimates for unactivated steroid receptors, which have ranged between 270,000 and 450,000 molecular weight [49]. Steroid receptor proteins themselves range from a molecular weight of only 65,000 to 120,000 Da [49]. The functional role(s) for associated proteins in the unactivated receptor complex has not been well defined. Several studies have suggested that hsp 90 functions to repress the activity of receptors in the absence of hormone and thus hold receptors in an inactive state until they are needed. In support of this, receptor activation in vitro induced by hormone binding, warming, or treatment with NaCl, or activation produced through hormone binding in vivo, results in the loss of associated hsp 90. Activation is also accompanied by conversion of receptors from 8–10S to 4S and from the non-DNA binding to the DNA binding state [50,51]. The 55- to 59-kDa component may play a role similar to that of hsp 90, since activation also results in dissociation of this component [41–46]. It has been suggested that the 55- to 59-kDa protein(s) and hsp 90 bind as a unit to receptors, with the 55- to 59-kDa protein associating indirectly through binding to hsp 90 [43,44,46]. The hsp 70 component is of interest because it has been found to associate, at least for GR and PR, with both unactivated and activated forms of receptors [41,42,45]. This suggests a different binding mechanism and functional role for hsp 70. Recent studies with mouse GR have suggested that hsp 70 may function in the transport of receptors across nuclear membranes in the absence of hormone [45]. This is supported by the fact that GR was observed to be predominantly nuclear in some cell types and cytoplasmic in others, and nuclear localization was related to the presence of hsp 70 in the unactivated GR complex [45]. Hsp 70 has been shown to be involved in facilitating protein transport across membranes of mitochondria and endoplasmic reticulum, an action believed to be due to the ability of hsp 70 to promote the unfolding of other proteins [52,53]. Hsp 70 could have a similar function in facilitating the unfolding of steroid receptors during transport across the nuclear membrane.

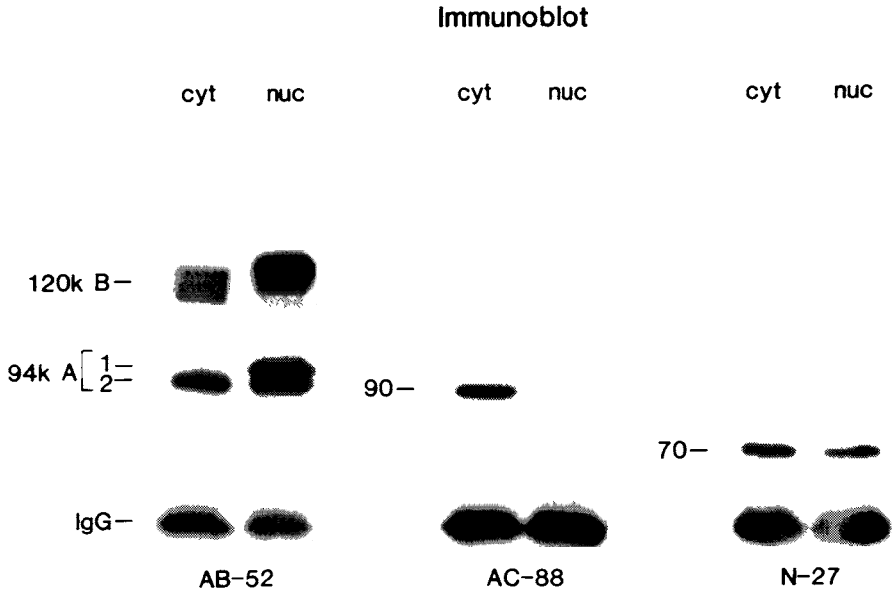
Association of hsp 90, hsp 70, and a 55-kDa protein with unactivated human PR isolated from human breast cancer cells is shown in Figure 2. T47D human breast cancer cells were incubated in culture for 48 hours at 37°C with [$^{35}$S]methionine to metabolically label PR and associated proteins to steady state. Cytosols were prepared and the unactivated PR complex was immunoaffinity purified with a receptor-specific MAb, AB-52 (specific for PR-A and PR-B), which had been coupled to an affinity bead (Affigel-10, Bio-Rad). The cytosols were prepared and incubated with MAb in the

continuous presence of sodium molybdate, since molybdate ion is known to stabilize the unactivated receptor complex in vitro. After extensive washing of the MAb beads to remove nonspecifically associated proteins, the immobilized, purified radiolabeled PR complex was eluted and analyzed by SDS-gel electrophoresis and autoradiography. As shown in Figure 2, the unactivated cytosol complex contains radiolabeled proteins of 120 and 94 kDa, which correspond, respectively, to the B and A forms of PR. The immune isolated complex also contains three other specifically associated [$^{35}$S]proteins of 90, 70, and 55 kDa. Parallel Western blot analysis with MAbs specific for hsp 90 (AC-88) and hsp 70 (N27) confirmed the identity of the 90- and 70-kDa bands as hsp 90 and hsp 70, respectively (Figure 2, right panel). We have not yet attempted to identify the 55-kDa component. Steady-state labeled receptors were also activated by hormone addition to intact cells (1 hour at 37°C), extracted from nuclei with high salt (0.5 M NaCl), and submitted to immune purification. As shown in Figure 2, activated nuclear receptors have lost associated hsp 90 and the 55-kDa protein but retain some hsp 70. The relative abundance of hsp 70 in the activated PR complex, however, was found to be reduced compared with the unactivated receptor, indicating that a partial dissociation of hsp 70 from receptors occurs during activation.

In addition to its proposed role as a repressor of receptor activity, several studies with GR have suggested that hsp 90 may have an additional more active function in regulating steroid binding activity. This is based on the findings that immune-isolated cytosol GR, stripped of hsp 90, exhibits no hormone-binding capacity [54]. Moreover, steroid binding activity can be restored by reassociation of hsp 90 with GR [55]. Additionally when GR are translated in vitro in a rabbit reticulocyte system, they are capable of both associating with rabbit lysate hsp 90 and of binding hormone with high affinity [56,57]. When translated in a wheat germ system, in vitro synthesized GR fail to associate with wheat germ hsp 90 and are unable to bind hormone [57]. This apparent requirement of hsp 90 for steroid binding may be less strict for PR than GR, since hsp-90-free PR is able to bind hormone, but at a reduced capacity [58].

The regions of GR and PR responsible for hsp 90 binding have been mapped by analysis of the ability of various receptor deletion and truncation mutants to form an association with cellular hsp 90. By these analyses, the hsp 90 binding region is contained within the steroid binding domain [59–62]. So far a more detailed mapping has been done with GR than with PR. Results have indicated that multiple sites within the steroid binding domain are necessary for hsp 90 binding [63,64]. The mapping data, taken together with the finding that hormone binding is influenced by the presence or absence of hsp 90, raises the possibility that hsp 90 may have an active role in maintaining the conformation or folding of the steroid binding domain necessary for high-affinity steroid binding.

It is interesting that not all steroid receptors in the family appear to have




the same ability to associate with hsp 90. For example, the thyroid receptor (TR) and retinoic acid receptors (RAR) translated in vitro under the same conditions as GR do not appear to have the capacity to bind hsp 90 [40,57]. TR and RAR also differ from the sex steroid and glucocorticoid receptors in that they bind tightly to DNA/chromatin in the nuclei of cells in the absence of hormone. This suggests that hormone-dependent activation of TR and RAR occurs by a fundamentally different mechanism that does not involve repression–derepression by hsp 90. As studies proceed we may see that steroid receptors will fall into subclasses based on their ability to interact with hsp 90 or with other proteins. Some receptors, such as PR and GR, appear to form fairly stable associations with hsp 90, as well as other proteins, and rely on assembly–disassembly of the heteromeric complex as a principal mode of regulating receptor activity. Other classes of receptors may interact less efficiently with hsp 90 or may not interact with the full complement of associated proteins. Some members of the family, such as thyroid and retinoic acid receptors, may not interact with hsps at all, raising the likelihood that this subclass has its activity regulated by an entirely different mechanism.

Dr. Toft's laboratory, working with chick PR, has for the first time been able to reconstitute an unactivated receptor-hsp complex in vitro [65]. This was accomplished by mixing rabbit reticulocyte lysates with purified hsp-free PR. This resulted in a temperature-dependent reassociation of hsp 90 and hsp 70, present in the lysate, with the purified PR. Development of this reconstitution system should facilitate future studies of how hsps regulate steroid receptor activity.

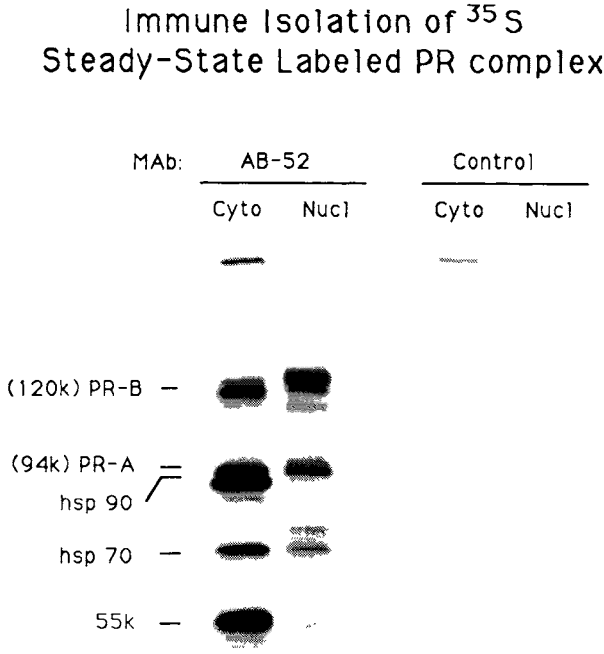



*Figure 2.* Protein composition of unactivated and activated forms of human PR. Above: T47D cells were metabolically labeled with [$^{35}$S]methionine and PR was immune isolated from either cytosol (nontreated cells) or from salt nuclear extracts of hormone-treated cells. Immune isolated complexes were analyzed for protein composition by SDS-PAGE and autoradiography. Left: Western blot of analysis of immune isolated PR complexes with MAbs specific for PR (AB-52), hsp 70 (N27), and hsp 90 (AC-88).

### 3. Hormone dependence of progesterone receptor binding to specific DNA sequences

A fundamental issue with respect to steroid receptor activation that remains unclear is whether hormone, in fact, promotes the binding of all classes of steroid receptors to their specific DNA sites. The activity of a gene regulatory protein can be controlled either at the level of DNA binding or through modification of the protein already bound to DNA.

Numerous studies dealing with this question have been published from several different laboratories and the results are quite conflicting. Many studies have shown that in vitro binding of receptors to their cognate DNA elements occurs efficiently in the absence of ligand and that ligand addition has no further effect on DNA binding [14,66–73]. A number of other studies have reported the opposite, finding that receptor binding to specific DNA is substantially enhanced or even is dependent on hormone [74–78]. In some instances, discrepancies in results may reflect true difference in modes of action, particularly for those receptor classes that do not interact

with hsp 90. As discussed above, RAR and TR do not appear to form associations with hsp 90 and have also been reported to bind to their specific DNA elements in a hormone-independent fashion [79,80]. This implies that RAR and TR bind constitutively to DNA and represent a subclass of non-hsp 90 binding receptors that are activated by a mechanism other than hormone promoting their binding to DNA.

It is interesting that unliganded TR bound to DNA appears to function as a repressor and hormone binding stimulates gene transcription by relieving the repression [80]. This suggests that hormone binding induces a structural modification of the TR molecule already bound to DNA. What that modification might be is not known. Whether the vitamin D receptor also falls into this subclass is not clear. Vitamin D receptors, like RAR and TR, bind tightly to nuclei in the absence of hormone, but a recent report showed that in vitro binding of vitamin D receptor to a vitamin D response element is dependent on hormone [81].

Discrepancies in the results obtained with receptors that do associate with hsp 90 (sex steroid and glucocorticoid receptors) are most likely due to differences in methodologies. For example, partial purification of receptors [72] or in vitro manipulations, such as warming or exposure to salts [14, 66,68], can result in dissociation of hsp 90 in the absence of ligand. This could allow binding of the unliganded receptors to DNA. Addition of ligand in this situation may have little or no effect on DNA binding and may not reflect the in vivo situation.

We have taken a slightly different approach to examine the question of the hormone dependency of human PR-DNA binding by comparing the influence of hormone when added to receptors both in vitro (cytosol) and in vivo (intact cell cultures). We have also used PR prepared as unfractionated cell extracts to minimize in vitro modification of receptors. Different forms of PR were prepared from T47D cells and compared for their DNA binding activity by a gel-mobility shift assay using the PRE of MMTV as target DNA. Receptor forms as shown in Figure 3 included (1) unoccupied 8–10S cytosol PR prepared from non-hormone-treated cells and stabilized by sodium molybdate, (2) cytosol PR prepared in the absence of molybdate and activated to 4S by hormone binding (synthetic analog R5020) in vitro followed by treatment with NaCl, and (3) 4S nuclear PR activated by binding of hormone in intact cells prior to their extraction from nuclei with 0.5 M salt. A [$^{32}$P]-labeled oligonucleotide derived from the PRE of MMTV was incubated with cytosol or nuclear extracts, and the resultant PR-DNA complexes were separated from free DNA by electrophoresis on nondenaturing polyacrylamide gels. As shown in Figure 3 (lane 1), the unactivated 8–10S cytosol PR failed to show any binding to the PRE oligonucleotide. We were able to induce a low but detectable amount of DNA binding by binding of R5020 to cytosol PR and subsequent treatment with NaCl to convert PR from 8–10S to 4S. This results in some PR binding to DNA, as evidenced by reduced mobility of the PRE-oligonucleotide

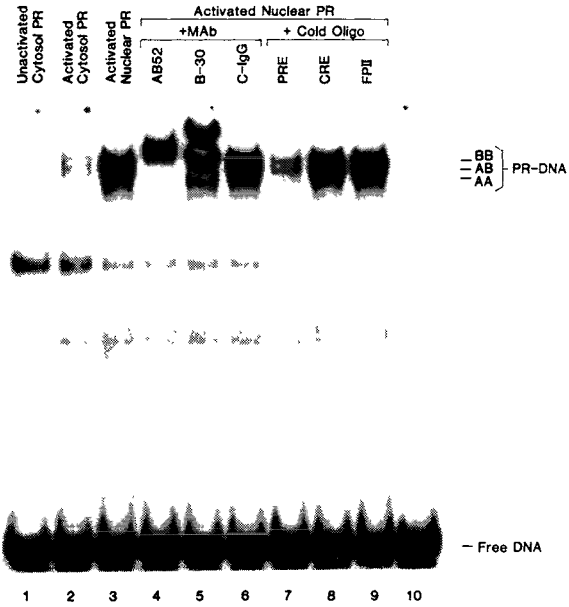



*Figure 3.* Binding of human PR to DNA is hormone dependent. Binding of different receptor forms to an end-labeled PRE oligonucleotide was analyzed by gel-mobility shift. PR in cytosol or nuclear extracts prepared from T47D cells (20 µl) were incubated with a [$^{32}$P]end-labeled double-stranded 28-bp synthetic oligonucleotide that corresponds to the most distal (bp −189 to −162 from the transcription start site) HRE of mouse mammary tumor virus. DNA binding buffers and conditions for gel electrophoresis have been described [13]. After incubation for 30 minutes at room temperature, samples were submitted to electrophoresis on nondenaturing 5% polyacrylamide gels. Positions of free DNA and PR-DNA complexes are indicated. A nonspecific protein-DNA complex is present in the middle of the gel. BB, AB, and AA indicate positions of the three dimeric forms of PR. The addition of receptor-specific MAbs (AB-52, B-30) and an unrelated MAb, C-IgG, are shown in lanes 4–6. Competition with excess unlabeled PRE-DNA or two unrelated oligonucleotides is shown in lanes 7–9. Lane 10 is DNA only. (Reproduced from DeMarzo et al. [75], with permission.)

(Figure 3, lane 2). Treatment with salt alone, however, was just as effective in inducing DNA binding as salt plus hormone (not shown). Activated nuclear PR formed by in vivo hormone addition displayed a substantially higher level of DNA binding (Figure 3, lane 3). In multiple experiments, in vivo activated PR was found to exhibit a five- to sevenfold higher DNA binding activity over that of cytosolic PR activated in vitro. The other lanes in Figure 3 illustrate the specificity of the reduced-mobility DNA complexes (lanes 4–10). The presence of PR in the upshifted [$^{32}$P]DNA complex is demonstrated by the further mobility shift produced by the addition of receptor-specific MAbs (AB-52 and B-30). Specificity of PR for PRE-DNA over that of nonspecific DNA is shown by the effective competition obtained with excess unlabeled PRE-DNA and the lack of competition with two unrelated oligonucleotides (Figure 3, lanes 7–9).

The above results demonstrate that human PR in the absence of hormone have little ability to bind to specific DNA under conditions that maintain the heteromeric 8–10S receptor complex. Hormone alone was unable to induce PR-DNA binding when added to cytosol, presumably because hormone binding in vitro also did not dissociate hsp 90 (not shown). Treatment with salt was needed to dissociate hsp 90, and this did result in induction of some PR-DNA binding. However, cytosol-hormone complexes, even when stripped of hsp 90, exhibited relatively low DNA binding activity. Thus, with cytosol PR we were unable to demonstrate an effect of hormone on DNA binding above that obtained by treatment with NaCl alone. A hormone effect on PR-DNA binding, independent of salt dissociation of hsp 90, was observed when hormone was added in vivo prior to receptor extraction from cells. In-vivo-activated PR exhibit a substantially higher DNA binding activity than salt-activated cytosol-R5020 complexes. This is despite the fact that both forms of receptors are dissociated from hsp 90. These results indicate that hormone binding in vivo produces a modification(s) of PR required for maximal DNA binding that is not mimicked by hormone binding in vitro. Results also demonstrate that hsp 90 dissociation is necessary but not sufficient for maximal activation of DNA binding activity. Thus our data support a model for receptor activation in which hormone does play an active role in promoting the high-affinity binding of human PR to specific DNA sequences.

The higher DNA binding activity of in vivo activated nuclear PR over that of PR activated in cytosol also suggested to us the possibility that nuclei contain a factor(s) that facilitates PR-DNA binding. We have shown that salt extracts of nuclei prepared from both hormone-responsive and nonresponsive cells contain a protein(s) that enhances the binding of cytosol PR to DNA [74]. This suggests that binding of human PR to specific DNA may be dependent both on hormone and nuclear accessory proteins. Nuclear accessory proteins that facilitate receptor-DNA binding have also been described for estrogen receptors [82], glucocorticoid receptors [83], thyroid receptors [84,85], and vitamin D receptors [81].

## 4. Progesterone receptor dimerization

Protein dimerization is emerging as an important regulatory mechanism for eukaryotic transcription factors. Since DNA response sequences often exhibit dyad symmetry, protein dimers are theoretically more suitable for DNA recognition than are protein monomers. Dimerization can provide a mechanism for increasing the affinity of a protein for specific vs. nonspecific DNA by creating more points of attachment per protein molecule than provided by a protein monomer. Dimerization potentially increases the effective protein concentration, since binding of one subunit will place the

other subunit in the vicinity of the DNA recognition site. Also, the spatial conformation of a protein dimer may be instrumental in providing the correct angular orientation between hydrogen bond donors and acceptors of protein and DNA [86]. Several eukaryotic transcriptional activators have been shown to form dimers both in solution and when bound to DNA. Dimerization in solution has also been shown in some instances to be a key regulatory step controlling DNA binding activity [87,88].

Several studies have now demonstrated that activated steroid receptors dimerize in solution in the absence of DNA and also exist as dimers when complexed to their cognate DNA recognition sites. This has been shown for estrogen receptors [14,76,89–91], glucocorticoid receptors [92,93], and progesterone receptors [13,15,75,94,95].

A commonly used technique to determine whether a protein binds to DNA as a dimer is to perform gel-mobility shift assays with protein size variants of two different lengths. If the protein binds to DNA as a dimer, three mobility protein-DNA complexes are expected: the fastest and slowest complexes represent homodimers of the long and short versions of the protein, and the intermediate complex represents a heterodimer composed of one long and one short subunit. This technique developed by Hope and Struhl in studies of yeast transcription factors [96] has been used to demonstrate that estrogen receptors [76,89] and glucocorticoid receptors [92] bind as dimers to their specific DNA recognition sequences. We have been able to exploit the fact that PR in T47D cells are naturally expressed as two different-sized proteins, PR-A and PR-B, to also demonstrate by gel-mobility shift that PR binds to specific DNA as a dimer [13].

As illustrated in Figure 4A, three distinct PR-DNA complexes are formed when gel-mobility shift assays are conducted with nuclear extracts containing both the A and B forms of human PR. This is consistent with PR complexing to PRE-DNA as dimers composed of the possible subunit combinations between PR-A and PR-B (AA homodimers, AB heterodimers, and BB homodimers). In further support of this, the intermediate mobility complex is more abundant than the other two. This is consistent with the formation of AA, AB, and BB dimers in the ratios of 1:2:1 predicted by binomial distribution. Moreover, the addition of an MAb reactive with both A and B forms of PR (AB-52) produced a further shift in the mobility of all three DNA complexes, indicating the presence of both PR-A and PR-B. The addition of a PR-B-specific MAb, B-30, produced a further shift of only the two upper complexes, indicating that no PR-B is present in the fastest mobility complex (Figure 4A). Also, when gel-shift assays were performed with nuclear extracts that contained PR-A alone (by depletion of PR-B with the B-30 MAb), only the fastest mobility DNA complex was evident. This complex was further shifted by the addition of the AB-52 MAb, but not by the B-30 MAb, indicating that it, in fact, contains PR-A subunits only (Figure 4B). The ability of human PR to bind DNA as three dimeric

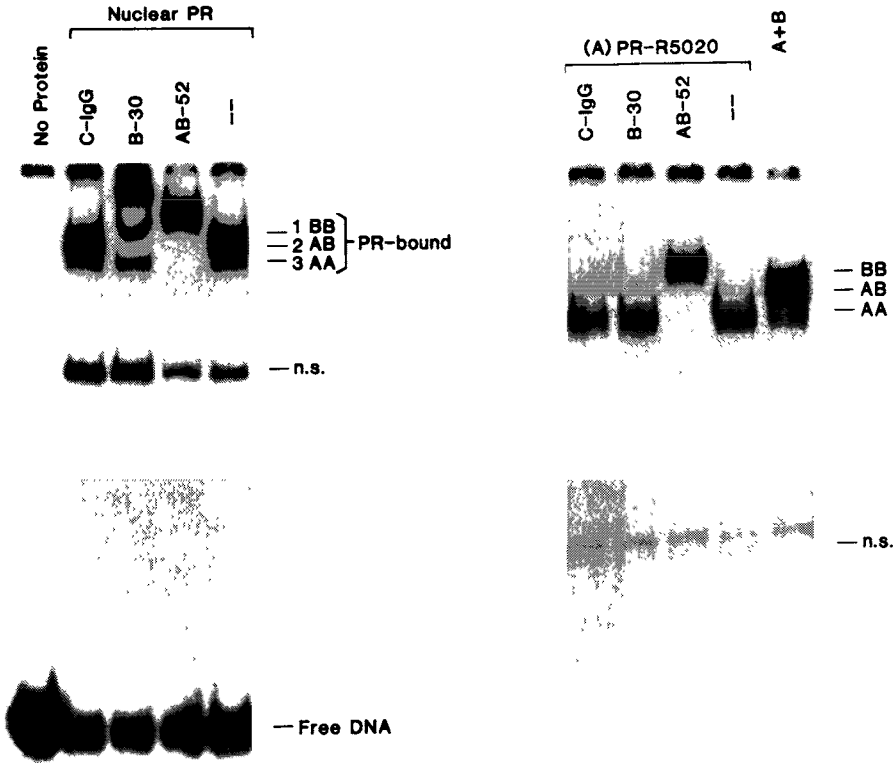



*Figure 4.* Human PR binds to specific DNA as a dimer. Left: Gel mobility shift assay with in vivo activated nuclear PR in the presence or absence of different MAbs, AB-52, B-30, and control IgG. Right: Gel-mobility shift with nuclear extracts containing PR-A alone or both PR-A and PR-B (A + B). Nuclear extracts obtained from R5020-treated T47D cells were depleted of PR-B (and PR-B/PR-A dimers) by passage over an affinity resin coupled with the PR-B-specific MAb, B-30. Note that free DNA was run off the gel. n.s. = nonspecific protein-DNA complex. (Adapted from El-Ashry et al. [13], with permission.)

species composed of AA, AB, or BB subunits has also been confirmed by Meyer et al. [15] by the use of vectors that separately express human PR-A and PR-B.

Although gel-mobility shift experiments demonstrate that PR are complexed to specific DNA as dimers, they do not address whether PR dimerization occurs in solution in the absence of DNA. To explore this question, we have again taken advantage of the fact that PR are expressed in approximately equal amounts in T47D cells as two different-sized proteins. If receptor dimerization between the A and B forms of PR were to occur in the absence of DNA, then immune isolation of cell extracts with the PR-B-specific MAb (B-30) should result in coisolation of some PR-A. To examine this, an immune coisolation assay was developed that involves first performing immune isolations with the B-30 MAb [75]. The isolated

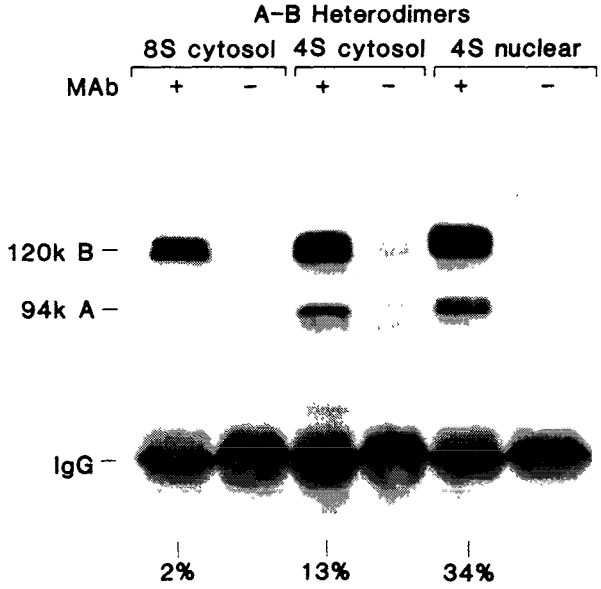



*Figure 5.* Immune coisolation assay to detect PR-A·PR-B dimers in the absence of DNA. The native forms of PR indicated were immune isolated (using protein A Sepharose as an immunoabsorbant) with the PR-B-specific MAb, B-30 (+), or a control, unrelated MAb (−). Immunobilized PR complexes were then extracted from protein A Sepharose and analyzed for the presence of both PR-A and PR-B by Western blot with MAb AB-52. The ratio of PR-A to PR-B in each isolated complex was estimated by direct scanning (Bioscan Inc.) of the counts of [$^{35}$S]protein A bound to PR bands and is expressed as the amount of PR-A as a percent relative to PR-B. (Adapted DeMarzo et al. [75], with permission.)

receptor complexes are then analyzed by Western blot with a different MAb, AB-52 (A and B specific), for the presence of both PR-A and PR-B.

This assay was applied to the same three PR forms analyzed in Figure 3 for DNA-binding activity. As shown in Figure 5, little or no PR-A was found to coisolate with PR-B in the unoccupied 8–10S cytosol PR complex. However, significant amounts of PR-A coisolated with PR-B when the assay was performed with either 4S in-vitro-activated cytosol PR or 4S in-vivo-activated nuclear PR (Figure 5). These experiments provide direct evidence that activated human PR are capable of forming stable PR-A · PR-B oligomers in solution in the absence of DNA. This immune coisolation assay does not distinguish, however, between a PR-A · PR-B heteromeric complex containing dimers or higher order multimers, nor does it detect the presence of homodimers containing either PR-A or PR-B alone. However, the generation of three PR-DNA complexes by gel-mobility shift assay (see Figure 4) favors that the PR-A · PR-B oligomers detected by the immune coisolation assay represent heterodimers composed of one subunit of PR-A and one of PR-B. It also follows that the random association of PR-A with

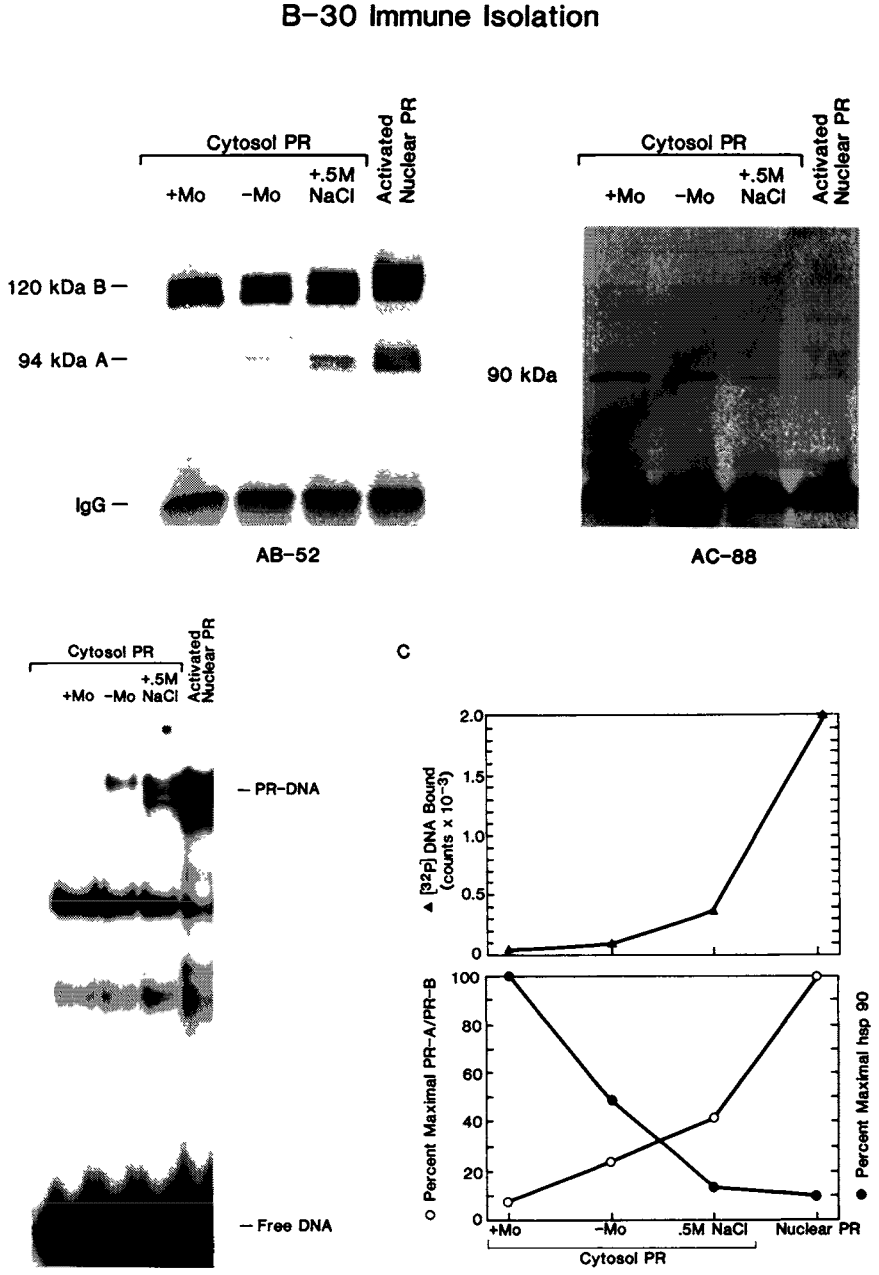



*Figure 6.* Dissociation of hsp 90 is correlated with the formation of PR-A·PR-B dimers in solution and with PR binding to DNA. A: Cytosol PRs from non-hormone-treated cells were prepared in the presence or absence of sodium molybdate (Mo) or were incubated for 4 hours at 4°C with 20 mM R5020 followed by the addition of 0.5 M NaCl and incubation for another 1 hour at 4°C. Activated nuclear PR was prepared as a salt extract of isolated nuclei after

PR-B in solution to form AB heterodimers would also allow the formation of AA and BB homodimers. It was of interest that we were unable to detect AB heterodimers with the unactivated 8–10S cytosol PR. This indicates that PR-A and PR-B exist as separate 8–10S complexes, each associated with hsp 90. This confirms earlier reports from Toft's group working with chick PR in which they were able to separately purify 8S PR-A and 8S PR-B complexes from molybdate-stabilized cytosols of chick oviduct [97].

The failure of unactivated cytosolic PR to form PR-A · PR-B dimers (Figure 5) suggested that the ability of PR to dimerize in solution might depend upon the presence or absence of hsp 90. To examine this further, receptors were prepared with varying amounts of associated hsp 90 and were then assayed for their ability to assemble into PR-A · PR-B dimers in the absence of DNA. The specific immune coisolation assay was performed as above, except that B-30 isolated complexes were analyzed by Western immunoblot for the presence of both PR-A (MAb AB-52) and hsp 90 (MAb AC-88). As shown in Figure 6A, an inverse relationship was observed between the presence of hsp 90 in the B-30 isolated complex and the extent of PR-A · PR-B dimerization. Molybdate-stabilized cytosol PR contained high amounts of hsp 90 and no detectable PR-A · PR-B dimers, while treatments that lead to a progressive dissociation of hsp 90 from receptor resulted in a parallel increase in the extent of PR-A · PR-B dimerization. When each of the same PR samples were analyzed by gel mobility-shift assay (Figure 6B), DNA-binding activities were found to be directly correlated with the ability of each receptor preparation to dimerize in solution (compare 4A and 4B).

Quantification of dimerization, hsp 90 association, and DNA binding by direct scanning of Western blots and dried mobility-shift gels (Figure 6C) illustrates that the abilities of PR to dimerize in solution and to bind to DNA are directly related and that both of these activities are inversely related to the presence of hsp 90. These data support the conclusion that dimerization in solution is an important regulatory step required for PR recognition of specific DNA sites. The inverse relationship observed between the presence of hsp 90 in the PR complex and the ability of PR to dimerize in solution supports the hypothesis that hsp 90 may repress DNA binding by blocking dimerization.

There is growing evidence that dimerization in solution in the absence of DNA may be a general feature in the steroid receptor family. Indirect

incubation of intact cells with R5020 for 1 hour at 37°C. Equal amounts in picomoles of each PR preparation were immune isolated with B-30. The isolated complexes were then divided into equal parts and analyzed by Western blot assay with AB-52 for the detection of PR-B and PR-A (left panel) or with AC-88 for the detection of hsp 90 (right panel). B: Gel mobility-shift assay of the same cytosol and nuclear PR preparations. C: Western blots (A) and the mobility-shift gel (B) were directly scanned for radioactivity (BioScan 200) to quantitate the relative amounts of associated hsp 90, the extent of A-B dimers formed, and DNA binding activities. (Reproduced DeMarzo et al. [75], with permission.)

evidence, based on kinetic and hydrodynamic properties, that activated ER are capable of forming stable dimers in the absence of DNA has been available for some time. This is supported by studies showing positive cooperativity of estrogen binding to ER [90] and to the behavior of activated ER on density gradient centrifugation and by gel filtration [91]. Immune coisolation of truncated ER with an antibody that recognizes an epitope present only in the full-length molecule has provided more direct evidence for the existence of ER dimers in the absence of DNA [89]. Detection of dimers in solution has been more difficult with other classes of steroid receptors, possibly because they are inherently less stable than ER dimers. As evidence for this, purified activated GR have been reported to form homodimers in solution that are stable to gel filtration but tend to dissociate into monomers during density gradient centrifugation [93]. Interestingly, maintenance of 6S GR dimers during gradient centrifugation was shown to require stabilization by either chemical crosslinking or the addition of DNA [93]. Other evidence for PR dimerization in the absence of DNA comes from studies on the localization of nuclear translocation sequences in the rabbit PR. Consistent with oligomerization occurring prior to DNA binding, ligand-induced nuclear uptake of a translocation-defective PR mutant was found to occur when cells were cotransfected with wild-type PR [94]. Rodriguez et al. [95] have demonstrated by chemical crosslinking and nondenaturing gradient gel electrophoresis that separately purified chick PR-A or PR-B are capable of forming stable homodimers in the absence of DNA.

The report by Fawell et al. [89] has provided the most definitive study as yet to directly address the question of whether steroid receptor dimerization in solution is a requirement for recognition of site-specific DNA. By analyzing various deletion and site-directed mutants, they have identified a conserved region of heptad-repeat hydrophobic residues in the steroid-binding domain of ER that is responsible for mediating dimerization in solution. They also demonstrated a direct correlation between the ability of ER mutants to form dimers in solution and their ability to bind to estrogen response elements. These results support the hypothesis that ER dimerization largely controls DNA binding activity. Our biochemical analyses revealing a positive correlation between the ability of different PR forms to dimerize in solution and their ability to bind to specific DNA sites (Figures 5 and 6) are also consistent with a model in which dimerization is a critical factor controlling PR-DNA binding activity.

Based on our studies and the studies of others, we propose that activation of PR-DNA binding is a hormone-dependent, multistep process. A working model is depicted in Figure 7. In the absence of hormone, PR are associated with hsp 90 and perhaps other proteins to form an inactive 8–10S complex with PR-A and PR-B, each existing as separate receptor–hsp 90 complexes. We believe that hsp 90 functions to repress DNA binding activity indirectly through blocking dimerization surfaces (ridged structures in Figure 7)

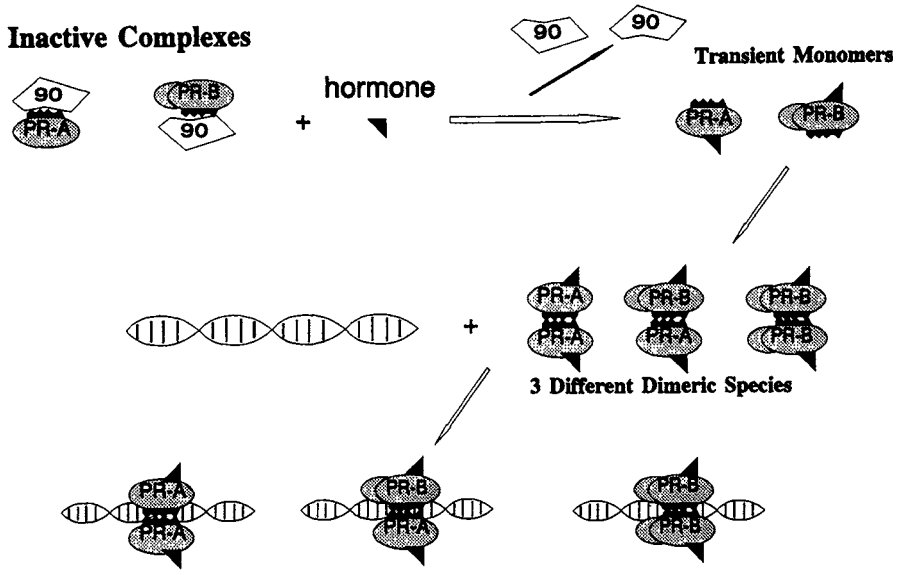



*Figure 7.* Proposed mechanism for activation of PR-DNA binding.

and not by directly masking the DNA binding domain. Hormone binding promotes dissociation of hsp 90, resulting in the formation of transient PR monomers with exposed dimerization surfaces. This is followed by formation of stable dimers as an intermediate step that occurs prior to binding to DNA. The dimeric form of PR is then competent to recognize specific dyad symmetrical DNA sequences.

An interesting aspect of proteins that use dimerization to control DNA-binding function is the formation of heterodimers as a mechanism to expand the functional diversity of a limited number of regulatory proteins [98]. This phenomenon has now been described for several gene regulatory proteins, including studies suggesting that thyroid receptors and retinoic acid receptors may be capable of forming functional heterodimers [99]. We predict that human PR are capable of forming the three dimeric species shown in Figure 7 as a mechanism to create novel receptor molecules, each with somewhat different functional activities and target gene specificities.

## 5. Progesterone receptor phosphorylation

Development of specific antibodies to steroid receptors has allowed definitive demonstration that they are phosphoproteins. PR from several sources have been shown to be phosphoproteins by their immunoprecipitation from cells or tissues incubated with [$^{32}$P]orthophosphate. Similar experiments have demonstrated that receptors for glucocorticoids, estrogens, Vitamin

$D_3$, androgens, and thyroid hormone are also phosphoproteins. For the most part, phosphoamino acid analyses of immune isolated [$^{32}$P]receptors have shown that modification occurs predominantly on serine residues [100, see review and references therein]. Some steroid receptors have also been described to be phosphorylated in the absence of hormone and to undergo increased phosphorylation upon in vivo addition of hormone. This has been observed with ER [101,102], GR [103], vitamin $D_3$ receptors (VDR) [104,105], AR [106], and PR from human [107], rabbit [108], and chicken sources [109,110]. In some cases, but not all, increased phosphorylation has been observed to result in a reduced mobility of the receptor protein on SDS-PAGE. For example, hormone treatment of ER produces multiple nuclear forms of 65 and 66.5 kDa, while untreated ER migrates as a single band at 65 kDa [101]. VDR also displays reduced electrophoretic mobility on SDS gels as a result of hormone-dependent phosphorylation [105]. With chick and human PR, both the A and B forms of receptor exhibit an increase in the apparent molecular weight on SDS gels in response to hormone binding in vivo. This has been shown to be due to phosphorylation, since the upshift on SDS gels can be reversed by treatment with phosphatase [111,112]. It is also of note that human PR-B exhibits molecular weight heterogeneity on SDS-PAGE, even in the absence of hormone, and this has also been shown to be due to phosphorylation [112].

The molecular weight heterogeneity of human PR on SDS-gels that is due to phosphorylation is shown in Figure 8. As detected by Western blot with AB-52, the unactivated cytosol PR-B migrates as a closely spaced triplet of bands at ≈120 kDa, while cytosol PR-A is a single band of ≈94 kDa. After activation by hormone binding in vivo, all of the triplet PR-B bands are upshifted, while only a portion of PR-A are converted to a slower mobility, resulting in the formation of a PR-A doublet (Figure 8, left panel). Alkaline phosphatase treatment of in vivo activated PR results in a partial reversal of the upshifted PR-B triplet but has minimal effect on PR-A (Figure 8, middle panel). This reversal is not seen in the presence of an alkaline phosphatase inhibitor. This illustrates that the molecular weight heterogeneity of PR-B is due to phosphorylation. That hormone binding is actually stimulating increased phosphorylation of PR is shown in the right panel of Figure 8. PR were immune isolated from T47D cells labeled to steady state with [$^{32}$P]orthophosphate, and isolated PR was then analyzed for incorporation of [$^{32}$P] by SDS-PAGE and autoradiography. Short-term hormone addition to cells (1 hour) resulted in a 2.5-fold increase in [$^{32}$P]labeling of both PR-A and PR-B. It should be noted that the increased [$^{32}$P] contained in PR-A and PR-B, due to hormone addition, exhibits a reduced mobility. This further demonstrates that the increased apparent molecular weight of PR observed on SDS gels is due to phosphorylation.

All steroid hormone receptors studied so far have been shown to be phosphorylated at multiple sites. Analysis of tryptic peptides has revealed that glucocorticoid receptor phosphorylation sites occur mainly in the

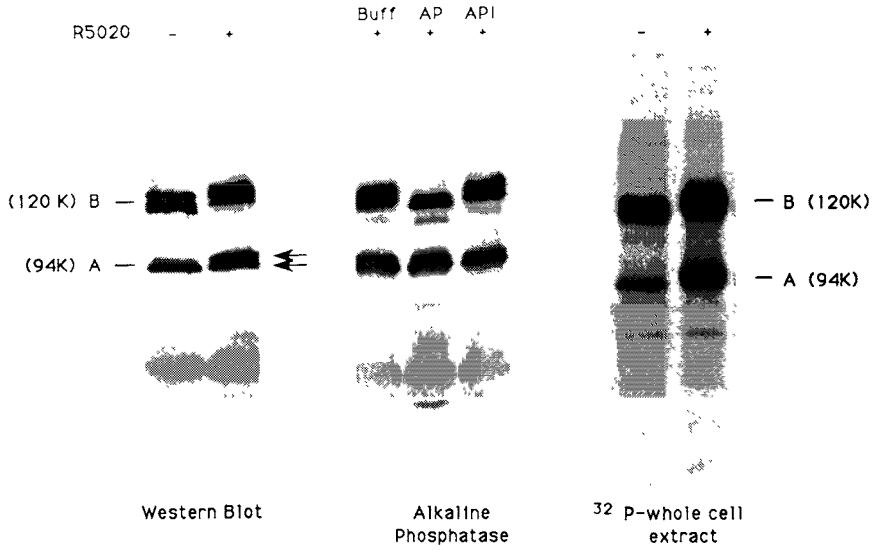



*Figure 8.* Increased phosphorylation of human progesterone receptors by hormone. Left: Unactivated cytosol PR (−R5020) and activated nuclear PR prepared from hormone-treated cells (+R5020) were immune isolated with AB-52 and detected by Western blot with the same MAb. Middle: Hormone-activated nuclear PRs were treated with or without alkaline phosphatase prior to immune isolation and Weatern blotting. Center: T47D cells were incubated 4 hours at 37°C with [$^{32}$P]orthophosphate and then treated for an additional hour at 37°C with or without R5020. Right: [$^{32}$P]labeled PR were then immune isolated from whole cell extracts and detected by SDS-PAGE and autoradiography.

amino-terminal transactivation domain [113] and in a region close to and partly encompassing the DNA binding domain [114]. Phosphopeptide analysis after chemical and protease digestion of chick PR has revealed that PR-A and PR-B show identical phosphorylation patterns and that all the phosphorylation sites are located amino terminal to the DNA and steroid binding domains. Hormone addition was found to increase the phosphorylation of existing sites, but no new phosphopeptides were found after hormone treatment [110]. However, higher resolution analysis of chick PR by gas-phase protein sequencing has revealed three hormonally regulated phosphorylation sites, two located in the amino-terminal domain, which were increased 1.5- to 2-fold upon hormone addition, and a third site located in the hinge region between the DNA and hormone binding domains. This third site was virtually absent without hormone and was greatly induced after progesterone treatment [111]. Phosphotryptic peptide analysis of human PR has shown that the peptides containing phosphorylated residues correspond to fragments from the first 595 amino acids of PR representing the amino terminus and the first 28 amino acids of the DNA binding domain [115]. Comparison of phosphopeptides from PR-A and PR-B revealed a

phosphorylated site unique to PR-B. Sheridan et al. [115], in this same study, also detected a difference between unactivated and activated receptors, suggesting the formation of new phosphorylation sites upon hormone binding. However, mapping of individual phosphorylation sites of human PR by amino acid sequencing has not yet been done.

The functional role(s) for steroid receptor phosphorylation remains poorly understood at present. Regulatory effects of phosphorylation are possible at several points in the pathway of steroid hormone action. The effect of phosphorylation on steroid binding activity has been studied most extensively with GR and ER. Several studies have shown that ATP may be required [116,117] for receptor to acquire ligand binding affinity. With ER, an increased affinity for estrogen has been reported to be the result of tyrosine kinase activity [118]. The effect of phosphorylation on steroid binding activity has not been studied thoroughly with other members of the steroid receptor gene family.

Regulation of receptor transformation (the ability of receptors to become tightly associated with DNA/chromatin) by phosphorylation has been examined. Sheridan et al. [107] concluded that phosphorylation of human PR does not regulate transformation, since the timing of hormone-dependent phosphorylation in cells follows that of tight PR binding to chromatin/DNA. Actual DNA binding experiments, however, were not done. Milgrom and coworkers concluded the same, since they found no differences in binding affinities of purified phosphoforms of rabbit PR for specific sequences in the uteroglobin gene [72]. These results conflict with the findings that phosphorylation of different steroid receptors in vivo and in vitro is accompanied by 8S to 4S conversion, loss of hsp 90, and increased binding to DNA cellulose [109,119,120]. In addition, studies from our laboratory have shown that nuclear human PR, activated by hormone in vivo, displays a substantially higher activity for binding to the HRE of the MMTV than PR activated in cytosol in vitro [75]. Since nuclear PR activated by hormone in vivo displays increased phosphorylation and activated cytosol PR does not [75], this provides a correlation between hormone-dependent phosphorylation and DNA binding activity. Thus the effect of phosphorylation on DNA binding in vivo and in vitro is at present uncertain.

A number of other eukaryotic transcriptional activators are phosphoproteins, and there is growing evidence that phosphorylation is critical for controlling transcriptional activity [121]. Perhaps the best example of this is CREB (cAMP response element binding protein). CREB can be activated by phosphorylation at a single site (Ser-133) by cAMP-dependent protein kinase A (PKA) [122]. It is believed that phosphorylation of CREB alters transcriptional activity without altering DNA-binding affinity, possibly by altering the tertiary structure of CREB and promoting interaction with proteins in the RNA polymerase II complex [123]. Several indirect studies have suggested that phosphorylation may be involved in

regulating transcriptional activity of steroid receptors. Denner et al. [124] working with chick PR reported that activators of cAMP-dependent protein kinase (8Br-cAMP) and inhibitors of protein phosphatases (okadaic acid) can mimic the effect of progesterone in mediating receptor-dependent transcription of a reporter gene in transient transfection experiments. However, a direct effect of these cellular modulators on PR phosphorylation was not shown. In similar experiments with human PR in T47D cells, we have obtained slightly different results. In the absence of hormone, 8Br-cAMP and okadaic acid had no effect on induction of target gene transcription (MMTV-CAT). However, simultaneous addition of hormone (R5020) and cellular modulators resulted in a two-threefold higher induction of reporter gene transcription than obtained with hormone alone [125]. Thus we observed an enhanced activation of receptors by hormone in the presence of cellular modulators of kinases/phosphatases but were not able to activate human PR in the absence of hormone, as was reported with chick PR [124]. The reason for the different results is not known. This could be due to true differences between chick and human PR or to experimental methodologies. Our studies utilized endogenous PR and a stably integrated reporter gene, rather than transient cotransfections of both receptors and reporter gene, as was done with chick PR studies.

Boyl and van der Walt have reported that activators of protein kinase C (PKC) increase phosphorylation of human PR [126]. In contrast, Rao et al. have reported no effect on PR phosphorylation with activators of either PKA and PKC [127]. Aronica and Katzenellenbogen [128] have observed that modulation of cellular kinases may play a role in regulating transcriptional activity of human ER. Activation of PKC pathways with phorbol esters has been reported to interfere with the ability of GR to mediate target gene transcription [129], and activators of both PKC and PKA were shown to increase phosphorylation in vivo of two naturally occurring sites in the thyroid receptor [130]. Thus it is possible that alteration in the activity of cellular kinases/phosphatases may exert an effect on receptor-mediated transcription, perhaps through modification of receptor phosphorylation itself by a cascade of phosphorylation–dephosphorylation events.

The functional role(s) for steroid receptor phosphorylation remains poorly defined. Since steroid receptors are phosphorylated at multiple sites, it is likely that more than one function can be regulated by phosphorylation-dephosphorylation. In several systems, increased receptor phosphorylation in response to hormone binding is seen as an early event in hormone action. Hormone-dependent phosphorylation, therefore, is likely to play an important role in the regulation of transcriptional activity. More definitive studies will require modification of individual receptor phosphorylation sites, either by site-directed mutagenesis or in vitro chemical methods, followed by an evaluation both in vitro and in vivo of the functional consequence of the modifications.

## 6. Modification of progesterone receptors by the hormone antagnist RU 486

The synthetic analog, RU 486, is a potent hormone antagonist for both glucocorticoids and progesterone, and is of most interest clinically for its antiprogesterone activity and potential as a chemical abortifacient. RU 486 is also of value as a compound to study steroid receptor mechanisms. As a general mode of action, RU 486 binds with high affinity to receptors (both GR and PR) and exerts its antagonist effects directly at the receptor level by competing for binding of the natural hormone agonist. Receptors occupied by RU 486 fail to become fully activated and thereby are unavailable to mediate the actions of the natural hormone [131–134]. The precise receptor mechanisms responsible for the action of RU 486 are controversial and remain largely unresolved. Based on studies from several laboratories, two hypotheses have been proposed. One proposes that RU 486 stabilizes the association between receptor and hsp 90, thereby impairing or blocking the ability of receptors to dissociate from hsp 90 and bind to DNA [135,136, and references therein]. The other hypothesis proposes that RU 486 is fully capable of promoting hsp 90 dissociation and receptor binding to DNA. The defect in receptor action is predicted to occur at a step after receptor binding to specific DNA sites [13,15,137,138]. It should be noted that the first hypothesis is based largely on studies with GR [139–143]. The preponderance of studies with PR, and particularly human PR, support the second hypothesis [13,15,137,138]. We have directly compared the effects of RU 486 on GR and PR in the same cell line (to eliminate cell-type specific differences), finding that RU 486 does have a stabilizing effect in vitro on the GR-hsp 90 complex. This is not evident, however, with PR under the same experimental conditions [144]. This indicates that RU 486 may exert its antagonist effects through GR and PR by somewhat different mechanisms. The most compelling evidence supporting the second hypothesis for the antiprogesterone action of RU 486 comes from gene cotransfection studies demonstrating that the PR-RU 486 complex is capable of binding to HREs in vivo. Milgrom and coworkers [138] cotransfected a constitutively active truncated PR (missing the steroid binding domain) and wild-type PR bound with RU 486. The presence of wild-type PR-RU 486 blocked the constitutive transcriptional activity of the truncated PR, indicating that the PR-RU 486 complex was exerting its inhibitory effect by competing for HRE binding sites in vivo. In a similar cotransfection experiment, Meyer et al. [15] found that human PR-RU 486 was able to block the activity of chick-PR-R5020 complexes. Since chick PR is not capable of binding RU 486, this study also demonstrates that the inhibitory action of the PR-RU 486 complex occurs at the level of competing for HRE sites in vivo [15].

To attempt to gain a better understanding of how RU 486 exerts its effects, we have compared physicochemical and functional properties of

human PR when complexed with hormone agonist (R5020) or with RU 486. The effect of RU 486 was examined at each step of the receptor activation process, up to and including binding to specific DNA sequences. We find that RU 486 behaves in vivo as a pure antagonist with respect to activation of target gene transcription. In a subline of T47D cells (clone B-11) containing a stably transfected MMTV-CAT reporter gene, RU 486 itself has no ability to activate gene transcription, and it effectively blocks the stimulatory effect of R5020 when given in a tenfold higher concentration [13]. Despite the inhibitory effect of RU 486 on receptor transcriptional activity, we also find that RU 486, both in vivo and in vitro, is fully capable of activating PR-DNA binding function. In fact, RU 486 does this somewhat better than R5020. For example, RU 486, both in vitro and in vivo, appears to have no stabilizing effect on the PR-hsp 90 complex. In vitro, the unactivated PR-hsp 90 occupied by either ligand exhibits the same sensitivity to salt dissociation [144]. Moreover, RU 486 added to intact cells has the same ability as R5020 to stimulate tight binding of PR to DNA/chromatin in nuclei [13,145]. RU 486 binding to PR also does not impair the ability of PR to dimerize in solution. In fact, by the immune coisolation assay used for detection of AB heterodimers [75], activated nuclear PR bound with RU 486 were found to be more efficient in forming dimers in solution than activated nuclear PR bound with R5020. Dimers formed in the presence of RU 486 were also found to be more resistent to salt dissociation, indicating that RU 486 induces a more stable PR dimer in solution than R5020 [146]. Consistent with the hypothesis that dimerization in solution controls DNA binding activity, we have also observed that PR bind more efficiently to specific PRE-DNA in the presence of RU 486. When receptor off-rates from PRE-DNA were measured by a gel-mobility shift method, PR were found to form a more stable complex with DNA in the presence of RU 486 than in the presence of R5020 [13]. We have also considered the possibility that although the PR-RU 486 complex binds tightly in vitro to PRE-DNA, it may not contact the exact same nucleotides in the PRE as the PR-agonist complex. Methylation interference footprinting was performed with unfractioned receptors in crude nuclear extracts, and PR activated by RU 486 was observed to make the same G contacts as R5020-activated receptors [147]. Sheridan et al. [115] have shown by [$^{32}$P]labeling of PR in T47D cells that RU 486 promotes an increase in PR phosphorylation. Therefore, RU 486 does not appear to exert its antagonist effect by preventing the increased PR phosphorylation that occurs with hormone agonist.

Binding of RU 486 to human PR does result in two observed alterations in structural properties that may provide a clue to explain how RU 486 works. First, PR-RU 486 complexes sediment at 6S on density gradients, compared with a 4S sedimentation for activated PR-R5020 complexes (Figure 9). This difference was first reported by Mullick and Katzenellenbogen [148], and has been subsequently observed by other laboratories [13,149,

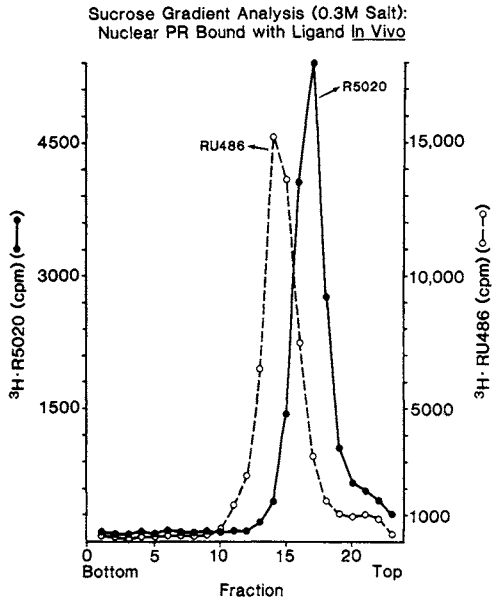



*Figure 9.* Sucrose density gradient centrifugation of PR complexed with RU 486 or R5020. T47D cells were incubated (1 hour at 37°C) with [$^3$H]R5020 or [$^3$H]RU 486. PR-hormone complexes were then extracted with 0.5 M NaCl from isolated nuclei and centrifuged on 5–20% on sucrose density gradients prepared in buffer containing 0.3 M NaCl. (Adapted from El-Ashry et al. [13], with permission.)

150]. It is of interest that the PR-RU 486 complex sediments as a 6S species whether receptors are bound with RU 486 in vivo prior to cellular extraction (Figure 9) or in cytosol in vitro [13]. Secondly, PR-DNA complexes on gel-shift assays exhibit a faster mobility in the presence of RU 486 than in the presence of R5020 [13]. As shown in Figure 10, each of the three PR-DNA complexes that correspond to AA, AB, and BB dimers have a faster mobility in the presence of RU 486. This difference in mobility on gel-shift assays was subsequently shown by Meyer et al. [15]. The molecular basis for the faster sedimentation of PR-RU 486 on density gradients and for the faster mobility of PR-RU 486 DNA complexes by gel-mobility shift is not known. We have preliminary data indicating that the faster sedimentation is not due to a more stable dimer induced by RU 486 being maintained during gradient centrifugation. Nor is it due to an associated protein not present in the PR-R5020 complex [146]. This raises the possibility that the faster sedimentation is due primarily to a different conformation change in PR induced by RU 486 binding. At first glance a faster sedimentation on density gradients, indicating a larger protein, is not consistent with a faster mobility by gel-shift, which indicates a smaller protein. However, these results could be reconciled if the PR-RU 486 complex were spherical in shape and PR-

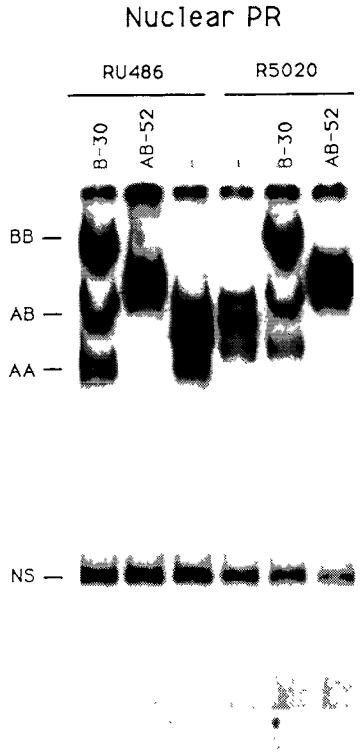



*Figure 10.* Comparison of the mobility of PR-DNA complexes in the presence of RU 486 or R5020. Nuclear PR-ligand complexes formed by the addition of either R5020 or RU 486 to T47D cells (1 hour at 37°C) were incubated with [$^{32}$P]-labeled PRE oligonucleotide and submitted to gel-mobility shift. The maximize the difference in mobility, free [$^{32}$P]DNA was run off the gel. To illustrate the protein composition of the PR-DNA complexes, the DNA binding reaction was performed in the absence and presence of MAbs AB-52 and B-30. (Adapted El-Ashry et al. [13], with permission.)

R5020 were more elliptical. A dense, more spherical molecule might exhibit both a faster sedimentation by density-gradient centrifugation and a faster mobility by nondenaturing polyacrylamide gel electrophoresis. This is highly speculative at this point, but is consistent with the available experimental data. A proposal for the mechanism of action of RU 486 is illustrated in Figure 11. RU 486 is predicted to promote efficient activation and binding of human PR to its specific DNA recognition sequences. As a result of an altered conformation induced by RU 486, the receptor bound to DNA does not interact efficiently with other transcription factors and thereby is unable to stimulate induction of transcription. If true, RU 486 should prove to be a valuable analog to study the receptor-protein interactions required for receptor-mediated gene transcription.

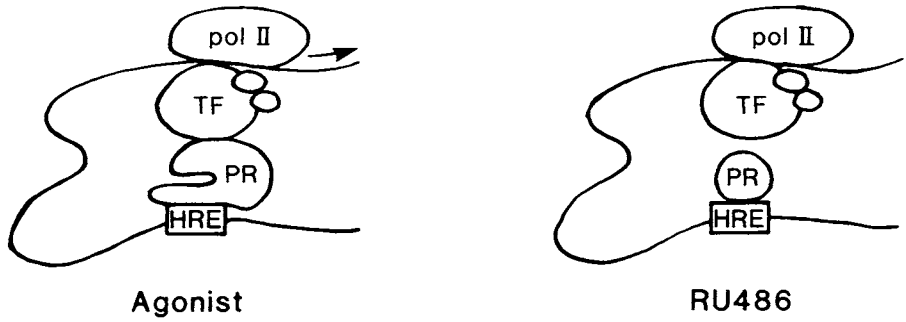



*Figure 11.* Proposed mechanism for RU 486 action.

## 7. Future directions

A major problem in breast cancer is the eventual progression of hormone-dependent tumors to the more aggressive hormone-independent state. Although escape from hormone control in many instances may be due to the loss of steroid receptor expression (≈30% of primary breast tumors and ≈50% of metastatic tumors are ER/PR negative), there is a significant subset of ER/PR-positive breast tumors (≈30%) that fail initial endocrine therapies with the antiestrogen tamoxifen. Moreover, many tumors that initially respond eventually develop tamoxifen resistance yet remain ER positive [151,152]. Now that the steroid receptor genes have been cloned and MAbs have been produced to study receptor proteins directly, there is a growing interest in determining whether variations in the steroid receptor molecule itself may be responsible for the loss of hormone responsiveness that occurs in many tumors.

Loss of hormone responsiveness in some breast tumors has been proposed to be due to mutations or truncations in the steroid receptor molecule itself [153]. In theory this could produce a constitutively active receptor that behaves like an oncogene by stimulating target gene transcription in the absence of hormone. Alternatively, dominant negative variants could be produced that inactivate coexpressed normal cellular receptors, or certain mutations could produce a functionally inactive receptor that retains hormone binding. These ideas are based on the fact that steroid receptor molecules are composed of discrete functional domains and that experimentally engineered deletions and other mutations have been shown to produce receptors with these kinds of altered biological activities [153, see review]. A number of variant receptor genes or mRNA for ER and PR have now been described to occur naturally that could potentially produce a steroid receptor with aberrant functional activities [154–158].

In addition to the production of mutant or truncated steroid receptors, what is the possibility that changes in phosphorylation states of receptors in certain breast tumors could create a constitutively active receptor with

oncogenic-like properties? How might steroid receptor phosphorylation be altered in tumor cells? Oncogenes that transduce signals across cell surface receptors to activate kinase pathways, or those oncogenes that possess kinase activities themselves, could act in a pleiotropic manner to affect phosphorylation of steroid receptors and thereby alter the sensitivity of a cell to steroid hormone in an epigenetic fashion. In support of this, oncogene activation has been linked to loss of hormone responsiveness. For example, stable transfection of MCF-7 cells with h-*ras* results in abrogation of estrogen growth dependence of MCF-7 cells in vivo [159]. ER levels in *ras* transformed cells were found to be comparable to that of wild-type MCF-7. They also bind hormone normally and induce at least one estrogen regulated gene, PR. No other analysis of the ER protein in MCF-7 *ras* cells has been done [159]. In other studies, both h-*ras* and v-*mos* transformed cells exhibit decreased sensitivity to glucocorticoids [129,160–162]. Reduced sensitivity to glucocorticoid may be due to an alteration in the glucocorticoid receptor itself that does not involve hormone binding. GR levels and hormone binding in v-*ras* and v-*mos* transformed cells were found to be normal, yet the ability of GR to mediate hormone-dependent transcription in these cells is dramatically diminished [129, 160–162]. Since v-*mos* is a cytoplasmic serine/threonine kinase, what is the possibility that it could modify receptors directly? H-*ras* (G protein homolog), on the other hand, could affect receptor phosphorylation indirectly through activation of signal transduction pathways.

In further support of the idea that altered cellular phosphorylation may contribute to steroid independence, there are now at least a few studies showing that activators of cellular kinase, as well as agents that inhibit protein phosphatases, can affect either the activity of steroid receptors or their phosphorylation [124,126,128–130]. These data, taken together with the link between oncogene activation and decreased hormone sensitivity, provide the rationale for the hypothesis that altered phosphorylation of steroid receptors may contribute to steroid independence in certain tumors. With the rapid progress that has been made in recent years in our understanding of the steroid receptor molecule plus the availability of molecular techniques and probes to study the receptor, answers to these relevant and interesting questions should be possible in the not too distant future.

## Acknowledgments

The authors acknowledge the contributions of past and present members of the laboratory: Dorraya El-Ashry, Angelo DeMarzo, Sergio Oñate, Blanka Kühnel, and Patricia Estes. We also acknowledge our collaboration on many of the studies described with Steven K. Nordeen's laboratory. We thank Nancy Hart and Clairene Mraz for skillful assistance in preparation of the manuscript, David O. Toft for providing the AC-88 MAb to hsp 90, and

William J. Welch for providing the N27 MAb to hsp 70. Studies were supported in part by Public Health Service grants CA41368 (DE), CA46938 (DE), and HD07430 (CB).

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PART V

# Differentiation and function of epithelium and stroma in the breast

# 17. Intermediate filament protein expression in normal and malignant human mammary epithelial cells

J. Taylor-Papadimitriou, R. Wetzels, and F. Ramaekers

## 1. Introduction

The proteins that make up the intermediate filaments of mammalian cells have in the last 10 years or so been defined in detail at the biochemical and structural level, while the profile of expression of these proteins has been monitored in a wide variety of cells and tissues. The various classes of intermediate filament proteins (IFPs), listed in Table 1, show considerable structural similarities but are antigenically distinguishable, so that monoclonal antibodies can be developed that exclusively recognize a single species. In studying the expression of IFPs at the cellular level, particularly in complex tissues, it is these monospecific antibodies that have proved to be extremely useful.

The cytokeratins, which form the intermediate filaments (tonofilaments) of epithelial cells, represent the most complex family of IFPs. In human epithelia, 20 soft-tissue keratins have been identified and classified [1,2]. On the basis of their molecular weight and isoelectric point determined by gel electrophoresis, the human keratins have been given the numbers 1–20. The numbers 1–8 form the keratin type II group of larger basic keratins, while the numbers 9–20 form the keratin type I group of smaller acidic keratins. In contrast to other IFP types, keratins must form heteropolymers of at least one keratin from each group to form a filament. As a result, epithelial cells express at least two different (and in most cases more than two) keratin polypeptides.

Initial studies on the expression of IFPs by different cell types suggested that the class of intermediate filament expressed were (with a few exceptions) specific for a particular cell or tissue type, as illustrated in Table 1 [3]. It also seemed that this tissue specificity was maintained in the change to malignancy, even when other parameters of differentiation were lost, making the IFPs extremely important antigens in tumor diagnosis. While both of these assumptions have proved to be generally valid, there are now sufficient examples in which representatives of more than one class of IFP have been found to be expressed in the same cell or tissue. These findings make it necessary to exercise caution in interpreting IFP expression to

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

*Table 1.* Intermediate filament types

| Gene family | Filament proteins | Tissue type |
|---|---|---|
| Type I | Keratins: small acidic | Epithelia |
| Type II | Keratins: neutral to basic | Epithelia |
| Type III | Vimentin | Mesenchyme |
| | Desmin | Muscle |
| | Glial filament protein | Astroglia |
| | Peripherin [95] | CNS stem cells |
| Type IV | Neurofilament proteins | Neurons |
| Type V | A- and B-type laminins | Most cells |
| Type VI | Nestin [96] | CNS stem cells |

define the differentiated and malignant phenotypes. Providing such caution is exercised, however, antibodies that are reactive with IFPs provide useful tools for approaching the difficult problem of cell characterization, in particular, in diagnostic histopathology and cytopathology.

The more detailed analysis of IFP expression, particularly in complex epithelial tissues such as the mammary gland, has been achieved primarily by using several monoclonal antibodies specific for a particular IF polypeptide, so that a range of epitopes responding differently to different routine tissue preparation and fixation techniques have been assayed. A comprehensive list of antibodies monospecific for individual keratins has been reported in a recent article by Lane and Alexander [4]. Since much of the analysis of intermediate filament expression discussed here depends on the use of monoclonal antibodies directed to the human family of proteins, and since many of these either do not react or show a different profile of reactivity with IFPs of other species, the discussion that follows will be confined to the human mammary gland. For some discussion of intermediate filament expression in the mammary epithelium of rodents, the reader is referred to our previous review article [5].

## 2. Basic features of intermediate filament structure and function

In most eukaryotic cells intermediate filaments constitute an important part of the intracytoplasmic matrix. This cytoskeletal component has been shown to interact with the plasma membrane at the periphery of the cell and with the nuclear lamina in the interior [6,7]. The diameter of these intermediate filaments, which has been estimated in electron microscopic studies, varies between 8 and 12 nm. The protein constituents of these filaments share the same basic structure, consisting of an alpha-helical rod domain of conserved secondary structure and size, flanked by nonhelical amino-terminal and carboxy-terminal domains of variable length, sequence, and chemical characteristics [7,8]. Althought the exact mechanism of filament formation is

not fully understood, the rod domain seems to play an important role in this process. The nonhelical domains of IFPs have, however, also been described to be involved in filament assembly. Several mechanisms have been proposed to explain the assembly of IF [9–11]. The most accepted theory states that assembly starts with the formation of the coiled coil from two monomeric protein subunits via side-to-side interactions of the rod domains. The forces involved in this reaction are mainly of a hydrophobic nature. As mentioned earlier it is assumed that, in contrast to most other intermediate filaments, keratin intermediate filaments are obligatorily composed of heterodimers (see below). It is believed that the second step in intermediate filament assembly is interaction between two dimers, resulting in a tetramer, followed by the formation of an octamer comprising two antiparalellely oriented four-chain complexes. In the resulting structure the nonhelical domains protrude from the filament backbone [12], although they are apparently also involved in filament assembly. These nonhelical end domains are the major sites of postsynthetic modifications, such as limited proteolysis and phosphorylation.

Phosphorylation of several types of IFPs has been shown to cause a reversible disassembly [13,14], while also specific $Ca^{2+}$-activated proteases, which cleave the IFPs at certain sites in the amino-terminal domain, may play a role in the assembly-disassembly cycle of intermediate filaments. Reassembly studies with intact and proteolytically digested IFP subunits demonstrated that the amino-terminal domains of several of the IFPs are necessary for normal filament assembly [15,16] and are involved in binding of the filaments to the plasma membrane [17,18]. The carboxy-terminal domain displays the highest variability, both in length and sequence, and might have a subunit-specific function. This domain is probably involved in the binding of intermediate filaments to the nuclear lamina [18,19].

The general consensus regarding cellular functions of intermediate filaments is that these cytoskeletal constituents perform a supportive role in the cell. This assumption is based mainly on the morphological appearance of intermediate filament networks within cells and the observation that these filaments form a link between nucleus and cell surface [20], interacting firmly with both structures (for example, through linkage to desmosomes). It does not, however, explain the diversity of subtypes found in functionally and morphologically different cell types, nor the finding that certain cell types can proliferate without containing a cytoplasmic IFP scaffold [21]. Functions of the individual IFP types at specific organ sites do, however, support a supposed structural role of these proteins. For instance the location of desmin in the z-disc of striated muscle and in the dense bodies of smooth muscle indicate the anchoring function to the contractile apparatus [22]. The neurofilaments are linked to neuronal microtubules in axons and may provide stability in this highly apolar cell. Vimentin filaments have also been implicated in RNA processing [23] and in interactions with nucleic acids [24].

### 3. Keratin expression in epithelial tissues

The framework on which our current understanding of keratin expression in epithelia is organized is substantially based on two concepts put forward in the early 1980s. Moll and colleagues [1] catalogued the human soft-tissue keratins by their relative size and change, initially giving them numbers 1–19, going from the largest (1) to the smallest (19) [1]. Only recently has keratin 20 been identified and characterized [2]. Thus, keratins 1–8 in the type II group are larger and have an isoelectric pH that is neutral to basic, while the type I keratins 9–20 are smaller and more acidic. Since keratins will only form filaments when type I and type II proteins are combined to form heteropolymers, epithelial cells contain at least one type I and one type II keratin, while some may contain as many as 10 different keratins.

The other important concept involves the pattern of expression of keratins in pairs and suggested a hypothesis linking the differentiated state of an epithelium with defined keratin pairs [25]. Although the details of this hypothesis are constantly under revision as new information appears, it has provided an important conceptual framework for the field, and most general predictions are still valid.

The distribution of keratins in epithelial tissues can best be considered by examining the three main types of epithelium in the human body (Figure 1), namely, (1) single-layered epithelium, which may be cuboidal or columnar and is referred to as simple epithelium; (2) stratified squamous epithelium,

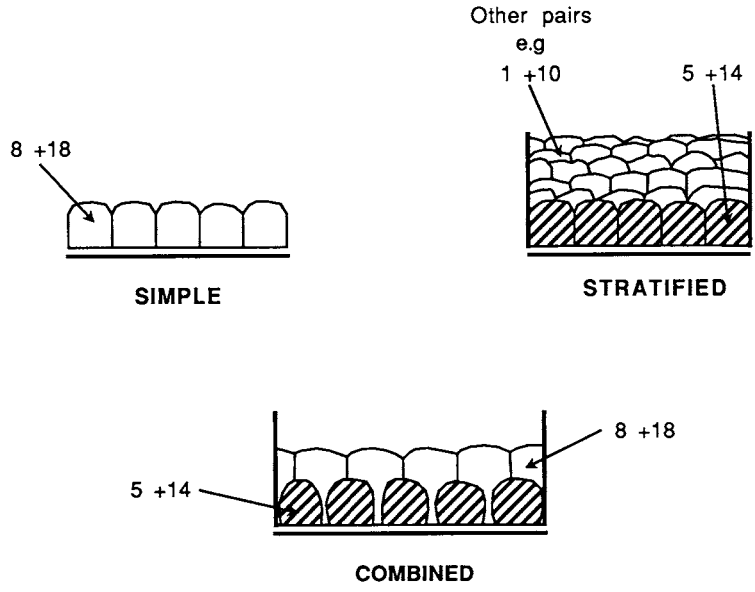


*Figure 1.* General principles underlying the expression of keratins in epithelial cells.

which may be subdivided into keratinizing (skin) and nonkeratinizing (e.g., oral or esophageal); and (3) mixed or combined epithelia (as in the mammary gland), characterized by a basal cell layer and a layer of columnar epithelium. In simple and combined epithelia, all cells are in contact with the basement membrane, while in stratifying epithelia only the basal cells have such contact.

Studies on keratin expression in the different kinds of epithelia have led to some general conclusions, namely, (1) the keratins expressed in the basal cells of both squamous and combined epithelial always include keratins 5 and 14 [2], (2) keratins 8 and 18 are always expressed in simple epithelia and in the columnar cells of combined epithelia, which have a similar topographical situation, i.e., they make contact with the basement membrane and have a free luminal surface on the apical side of the cell, (3) in the suprabasal cells of stratifying epithelia, additional pairs of keratins are expressed as the cells differentiate, and which pair is expressed depends on the particular tissue [25,27]. A considerable amount of diversity in keratin expression is grafted onto these basic ground rules. For example, simple epithelia and the columnar cells of combined epithelia may also express keratins 7, 19, and 20 [2,28–30], depending on the tissue. Also, the basal cells in stratifying epithelia or combined epithelia may express keratin 17 [31–33].

Until recently the 'simple epithelial keratins' 8 and 18 were thought to be restricted to simple epithelia and luminal cells of combined epithelia. Similarly, the 'basal keratins' 5 and 14 were thought to be restricted to stratifying epithelia and the basal cells of combined epithelia. While this is generally true, there are now reports of a less restricted expression of these keratins. Thus, low levels of expression of mRNA coding for the simple epithelial keratins has been detected in some stratified epithelial [34]. However, these keratins at best represent a minor component in these tissues. Also using monoclonal antibodies to these individual keratins, normal, and especially malignant, stratified epithelia have been shown to contain keratins 8 and 18 [35,36]. Similarly, keratins 5 and 14 can be found in some luminal cells of combined epithelia [5,33,37]. Keratin 19, although mostly found in simple epithelia and columnar cells of combined epithelia, can also be detected in some basal cells of both stratifying and combined epithelia [27,29,30]. Its expression has been reported to relate to the proliferation and/or differentiation in oral mucus [3] and in the mammary gland [39,40].

As indicated above, the epithelial compartment of the mammary gland is classified as a combined epithelium (see Figure 1). Two cell layers line the ducts and alveoli of the mammary tree, i.e., the basal or myoepithelial cells, which sit directly on the basement membrane, and the luminal or secretory epithelial cells, which line the lumen. Although the luminal cells rest on the basal cells, they do have processes that extend down to the basement membrane and are in contact with it.

Most of the data on which we base our knowledge of the expression of different keratins in complex epithelia are based on the use of specific monoclonal antibodies. In ascertaining whether a particular keratin is present or not, the importance of examining the tissue with several monoclonal antibodies and checking overall expression at the biochemical level cannot be overemphasized [for antibodies see 4]. This is because a particular epitope conformation may be lost or epitopes may be masked and thus reduced in their immunoreactivity after certain fixation procedures. This masking, when dependent on interaction with another keratin to make a filament, can also be specific to a particular tissue or differentiation stage [36]. Conversely, an epitope may only be detected when the keratin is bound to a specific partner (e.g., KA1 is only reactive with keratin 5 when complexed with keratin 14 [37,41]). Although this means that caution should be exercised in using antibodies to detect the expression of a particular keratin, the antibodies still remain invaluable and powerful tools for distinguishing cell phenotypes and, thus, for the study of differentiation in normal phenotypes and of malignancy. In the tissues and tumors of the mammary gland, an extensive panel of monoclonal antibodies have been used to study keratin expression, and in some cases biochemical analyses have been performed. For the sake of clarity, we will therefore define cell phenotypes by the keratins they have been shown to express, rather than by their antibody reactivity.

## 4. Structural origin of the mammary gland

The mammary gland, like other exterior secreting glands, is derived from the epidermis in fetal life. In its final structural form, as well as in its patterns of development, it belongs to the same tissue type as sweat glands, salivary glands, and lachrymal glands. Not surprisingly, therefore, the mammary gland also exhibits a similar keratin phenotype to that of sweat glands and salivary glands. We have previously reviewed some details regarding the development of the mammary gland [5]. Here we will mention, therefore, a few points that are worth emphasizing, bearing in mind that most of these studies have used nonhuman embryos. Studies in mouse embryos have shown that keratins are present from the earliest stages of embryogenesis, and the first intermediate filament proteins to appear are the mouse keratins Endo A and Endo B (equivalent to the human keratins 8 and 18); keratin 19 appears somewhat later around the time when the first vimentin-expressing mesenchymal cells appear [42–44]. A second point is that, based on data derived from the mouse, it is clear that the development of the mammary gland commences after the epidermis begins to stratify but when the periderm is still present. Thus, the mammary gland arises from a developing epidermis that consists of two cell types, that is, the keratinocyte precursors expressing epidermal-type keratins (human 5 and 14) and keratin

19 [45] and the periderm expressing simple epithelial keratins 8 and 18 as well as 19 [46].

## 5. Intermediate filament protein expression in the normal human adult mammary gland

Although there are other cell types in the mammary gland, the epithelial cells making up the mammary tree form the most complex component, and it is from these cells that breast cancers develop. Moreover, the profile of intermediate filament protein expression, particularly of the cytokeratins, is proving to be extremely useful in subclassifying apparently similar cells in the mammary tree. Beyond noting that the stromal and endothelial cells in the tissue express vimentin and the smooth muscle cells express desmin, we will confine our discussion to the mammary epithelial cells and to the epidermis around the nipple.

At the biochemical level, the keratins expressed by the human mammary gland (as detected by two-dimensional gel electrophoresis) were found by Moll and colleagues to be keratins 5, 7, 8, 14, 15, 17, 18, and 19 [1]. Of these, keratins 7, 8, 18, and 19 are commonly found in simple (nonstratifying) epithelial with 8 and 18, more or less 'diagnostic' of simple epithelia. Keratins 5, 14, 17, and 19 are found in stratified squamous epithelia, with 5 and 14 regarded as diagnostic of the stratified squamous cell types, such as the keratinocyte [26], and of the basal cells in combined epithelia. There is no biochemical evidence for the presence of the suprabasal keratinocyte differentiation keratins (i.e., 4 + 13, 6 + 16, and 1, 2, + 10) in the human mammary gland [33].

### *5.1. Distinction between basal and luminal epithelial cells*

As indicated earlier, it is evident that keratins 5 and 14 are expressed by all basal cells throughout the gland, while keratins 7, 8, and 18 are expressed by all luminal cells. Moreover, basal cells do not appear to express keratins 8 and 18. However, with regard to the basal-specific keratins 5 and 14, some staining of a subset of luminal cells in the TDLU has been seen with antibody KA1 to keratin 5 [33,37,41], and some expression of keratin 14 was detected in the luminal cells of the terminal and larger ducts using the antibodies LL001 [40] and LL002 to keratin 14. More recently, Wetzels and colleagues have used three different antibodies to detect keratin 14 expression in these luminal cells [47].

Other intermediate filament proteins that are differentially expressed by the two epithelial cell types are vimentin, and keratins 17 and 19. Until recently there was controversy regarding vimentin expression in the mammary gland. It is now clear, however, that vimentin is expressed, but it is only detected in the myoepithelial or basal cells [32,41]. Similarly K17

*Table 2.* Overview of expression of intermediate filament proteins by the different types of epithelial cells in the mammary gland

| Structure | Basal or myoepithelial cells | Luminal cells |
|---|---|---|
| Large ducts | Keratins 5,14,17 (7),(19)<br>Vimentin[a] | Keratins 7,8,18,19 (14),17[f] |
| Terminal ductal lobular units (TDLU) | Keratins 5,14,17[b] (7)<br>Vimentin[a]<br>Glial filament protein[c] (GFP)[f] | Keratins 7,8,18 19[d,f],5[e,f],17[e,f] |

[a] Varying degrees of intensity of staining reported between different parts of the mammary tree and between samples [32,41].
[b] Reported to stain fewer cells in the "lobular alveoli."
[c] Antibodies react with subpopulation surrounding TDLU [41].
[d] Luminal cells *not* expressing keratin 19 are a small subgroup.
[e] Occasional reaction seen with some luminal cells in TDLU [37]. TDLU = Terminal ductal lobular unit, which refers to the terminal duct and the lobules branching from it, as defined by Wellings et al. [52].
[f] A subset of cells are stained.
Brackets indicate weaker staining than seen in the other epithelial cell type

expression appears to be confined to the basal cells [32], although occasional staining in the luminal cells has been reported [33].

Keratin 19 is the smallest keratin and lacks the C-terminal variable amino acid sequence characteristic of other keratins and IFPs [48–50]. It can be found in both simple and stratified epithelial, and changes in the pattern and level of its expression may be related to differentiation in some cases [39,51]. It has been shown that keratin 19 can form heteropolymers with the type II keratins commonly expressed in simple epithelial cells (keratins 7 and 8) [15]. It is not clear, however, which type II keratin contributes to the K19-containing filaments found in some basal cells in stratified and combined epithelia. In the mammary gland keratin 19 is found predominantly in the luminal epithelial cells, where several keratin 19 antibodies have been shown to give very strong staining with most luminal cells. These antibodies do not stain the basal cells in the luminal duct and TDLU, but can give a weaker staining with basal cells in the large ducts. There is a subset of luminal epithelial cells in the TDLU that do not express keratin 19 (see below), which we believe to form a separate proliferative compartment and which may represent a specific phenotype in the luminal cell lineage (see next section). A summary of the intermediate filament proteins expressed by the luminal and basal epithelial cells in the mammary tree is given in Table 2. The fact that both keratin 19 and keratin 7 are weakly expressed in the basal cells of the large ducts suggests that, again, these two keratins may form a heteropolymer, as in the simple epithelial cells [15].

### *5.2. Subsets of epithelial cells defined by intermediate filament expression*

Much of the proliferation seen at pregnancy leads to the production of more terminal ductal lobular units (TDLU) and to further branching within these

units. Moreover, it is thought that it is from cells in this part of the gland that breast cancers develop [52]. The cells in these structures are, therefore, of particular interest. As can be seen from Table 2, antibodies to keratin 19, keratin 5, and keratin 17; to glial filament protein (GFP); and possibly to vimentin appear to define subsets of luminal or basal cells in the terminal ductal lobular units. Whether these subsets, in fact, represent differentiation stages in the lineages is not clear, since evidence supporting such a hypothesis, if available, is indirect. We have, however, attempted to obtain such evidence for the subset of keratin 19-negative luminal cells found in the TDLU.

**5.2.1. Keratin-19-negative luminal epithelial cells.** The keratin-19-negative luminal cells are seen in the terminal ducts and lobules in the region where the initial changes leading to malignancy are thought to occur [52]. Although a minority cell population, the keratin-19-negative cells proliferate well in culture and are found in increased numbers in benign tumors. They are generally, however, not found in the invasive component of breast cancers [51] (see Section 6). Of great interest is the observation that the keratin-19-negative luminal cells only appear at puberty when ductal branching occurs [53], suggesting that they are associated with normal proliferation in normal development. They appear to be less differentiated than the keratin-19-positive luminal cells, since those TDLUs that contain a high proportion of keratin-19-negative luminal cells do not make a secretory component. Also, although such lobules with many keratin-19-negative luminal cells are very rare in the lactating gland, when they are identified they do not secrete casein. Taken together, the above observations support the hypothesis that the keratin-19-negative luminal cell in the TDLU is a transit cell that may be a precursor to the more differentiated keratin-19-positive cell [54].

**5.2.2. Keratin-5-positive luminal epithelial cells.** The subset of luminal cells staining positively with the KA1 antibody that is directed to keratin 5 in conformation with keratin 14 is also located in and around the TDLU [33,37,41,55]. Staining of developing gland and lactating tissue has not been reported for this antibody. Conceivably this subset of cells represents cells emerging from the basal layer to differentiate into luminal cells. It would be important to stain serial sections with KA1 or other keratin 5 and/or 14 antibodies in parallel with antibodies to keratin 19 to see if the keratin-19-negative and keratin-5-positive cells were found in the same region and whether they were identical.

**5.2.3. Mammary epithelial cells expressing GFP.** In contrast to the keratin-19-negative and keratin-5-positive subsets described above, the cells expressing GFP are located in the basal layer, but again they are in the terminal ducts and TDLU. This observation is very recent [41], and one can only speculate on the implications. An attractive idea would be that they

might represent a pluripotent stem cell in the basal layer able to give rise to both basal and luminal cells.

**5.2.4. Mammary epithelial cells expressing vimentin.** Although it is clear that it is the basal cells of the mammary gland that express vimentin [32,41], it is not clear as yet what proportion of the cells is expressing this protein (together with keratins 5 and 14, and possibly GFP). Data from one of our laboratories suggests that vimentin expression is a feature of most basal cells (Taylor-Papadimitriou, unpublished observations), whereas Ramaekers and colleagues find a minority of cells stain positively with vimentin antibodies (Ramaekers, unpublished observations). It may be that the differences seen in different laboratories may be attributable to the use of different antibodies, fixation procedures, and the integrity of the samples. Since early studies reported no vimentin expression in any mammary epithelial cells, it is possible that the level is not as high as in mesenchymal cells, and inactivation of antigen, either through fixation or during storage, may readily affect detectability.

*5.3. Intermediate filament expression in nipple epidermis*

As indicated earlier, the breast develops from the embryonic epidermis when it begins to stratify, but when the periderm is still present, and both the keratinocyte precursors and the periderm express keratin 19. This may have some relevance to the fact that in the adult nipple epidermis, keratin 19 is expressed by some of the basal cells [30,53]. This is in contrast to other sites in the human body, where keratin 19 is not expressed in postnatal interfollicular adult epidermis. The keratin-19-positive cells in the nipple epidermis do not, however, express keratins 8 and 18, which are expressed by the luminal epithelial cells of the mammary tree. It is therefore the expression of keratins 8 and 18, and the lack of expression of typical keratinocyte keratins, that has led to the conclusion that the malignant cells in Paget's disease (which also express keratin 19) derive from the mammary epithelial cells and not from nipple epidermis [55–57].

## 6. Intermediate filament expression in breast tumors

It has been emphasized for some time by pathologists [58] that a major difference between benign and malignant tumors of the breast is that the former retain myoepithelial elements, while the latter do not, with the exception of the myoepithelial cells encircling the in situ component. Analysis of the profile of intermediate filament protein expression by benign and malignant tumors has largely confirmed this idea, and from initial studies it was thought that only keratins typical of the luminal mammary epithelial cells were expressed in invasive breast cancer cells. Recent studies

have, however, identified a small subset of malignant tumors that do contain a certain percentage of cells expressing intermediate filament proteins found mainly in the basal or myoepithelial cells in the normal gland. These recent observations are important, since they may give some clues regarding the definition of specific phenotypes in the cell lineages of the mammary gland.

### *6.1. Intermediate filament expression in benign tumors*

Benign tumors in the mammary gland form a heterogeneous group that, with the exception of fibroadenomas and papillomas, are not always classified in the same way in different laboratories but are usually collectively referred to as *fibrocystic disease*. All of them, however, retain some glandular structure, albeit distorted. In these structures, as with the normal gland, the luminal cells express the simple epithelial keratins 7, 8, and 18, and the basal cells express keratins 5, 14, and 17. On this background, however, some differences from the normal gland can be seen, mainly in the subsets of cells discussed above. In all except some papillomas, there is a marked increase in the number of luminal cells that do not express keratin 19. Also, in some cases of sclerosing adenosis, Dairkee and colleagues [59] have found that all the cells in the ducts stain with antibody 312C8-1 directed to keratin 14. This observation supports previous suggestions that while ductal hyperplasia (with the exception of sclerosing adenosis) represents proliferative lesions of the luminal epithelial cell component, sclerosing adenosis originates from the basal cells.

Vimentin expression in fibrocystic disease was first noted by Raymond and Leong [60], who found evidence for expression in 7 of 20 cases examined. The expression of vimentin and GFP have been examined in a small number of benign tumors by Gould and colleagues, who have found evidence for expression of these proteins in 14 of 23 and 18 of 23 cases, respectively [41]. Expression was mainly confined to the basal cells, with rare cases of positive staining being seen in some cells in a luminal position. These authors have shown that vimentin and GFP are coexpressed with keratins 5, 14, and 17. Moreover, the greatest number of GFP expressing myoepithelial cells were noted in some highly proliferative forms of fibrocystic disease, in other benign tumors, and in some in situ tumors.

One explanation that we favor for the apparent increase in the subsets of luminal and basal cells in benign tumours (as defined by their unique profile of intermediate filament expression) is that these do indeed represent different phenotypes in the cell lineage that proliferate and differentiate to form the functional luminal or basal epithelial cell. Benign tumors probably arise from the polyclonal growth of many cells going through a few division cycles, resulting in a heterogeneous tumor cell population, with an increased proportion of those phenotypes that have a high proliferative capacity. The fact that the subpopulation of breast cancers that contain vimentin or K14-expressing cells have a poor prognosis supports this view (see below).

*6.2. Intermediate filament expression in breast cancers*

Initial studies using monoclonal antibodies reactive with a single intermediate filament protein indicated that, as predicted from the pathology [58], the invasive cancer cells express the simple epithelial keratins characteristic of the luminal epithelial cells. Keratins 8 and 18 are uniformly expressed [5], as is keratin 19 in more than 90% of tumors [51,61]. Keratin 7 is usually seen in the invasive cells but can be lost, and is quite frequently absent from cell lines derived from tumors (see section 7). A more detailed examination of a larger number of tumors with panels of monoclonal antibodies has shown, however, that a subset of tumors do in fact contain cells that express intermediate filaments found in the basal or myoepithelial cells in the normal breast. Thus, Dairkee and colleagues [59] and Wetzels et al. [33,47] have found that keratin 14 was expressed with 12 of 118 and 10 of 116 cases, respectively, showing some positive staining. The antibody KA1 known to react with keratin 5, forming a heteropolymer with keratin 14, has also been found to stain a fraction of the cells in a subset of carcinomas [33,37,41].

There appears to be some difference between the staining of the various antibodies to keratin 14, with the antibody LL002 [62] giving fewer positive samples [33]. Possibly there may be masking of the LL002 epitope or, since the epitope is at the carboxy-terminal end of the molecule, it could be lost by proteolytic cleavage, as happens in the stratum corneum during differentiation of skin. In the study by Dairkee et al., only half showed a homogeneously positive staining, but these appeared to have a bad prognosis [63]. Of interest is the fact that keratin 14 was not found to be expressed in the lobular carcinomas examined.

Although vimentin was originally reported to be absent from breast cancers, there are now several reports of positive staining with antibodies to vimentin in a subset of breast cancers [60,64,65]. As with K14, vimentin does not appear to be expressed by lobular carcinomas [41,66] but is found predominantly in medullary and high-grade infiltrating ductal carcinomas. In the study by Domagala et al. [66], 35% of grade III tumors showed vimentin expression, while only 9% of grade II and no grade I tumors showed positive staining (total tumors analyzed = 214). Vimentin appears to be preferentially expressed in cancers with a low estrogen-receptor content and a high Ki67 defined growth fraction, p53 expression, and EGF receptor content [64,67,68]. Indeed vimentin expression appears to be associated with a poor prognosis, even in node-negative ductal breast carcinomas [67]. Antibodies to vimentin (and possibly to keratin 14) are therefore among the new tools for assessing prognosis in infiltrating ductal carcinomas. It is not clear why the discrepancies arose in detecting vimentin expression, since in some studies, notably those by Domagala et al., formalin-fixed, paraffin-embedded sections were used and the results agreed with previous studies using frozen sections. It will probably be important to establish standards for

tissue fixation and antibodies in order to make the assessment of prognosis by vimentin expression more widely available.

The observation that GFP can be detected in a subset of breast cancers (1 of 20 ductal, 1 of 7 lobular, 1 of 1 papillary) is very recent [41], and studies on the prognostic implication of this expression have not been published. However, the identification of tumors expressing K14, vimentin, or GFP, and the association of K14 or vimentin expression with ductal carcinomas or with a poor prognosis, is of great interest. It gives weight to the idea that the subsets of cells in the normal breast expressing these proteins do indeed represent precursor cells (stem cells or transit cells), which have a higher proliferative capacity than the differentiated luminal cell that develops from them and that forms the dominant phenotype in breast cancers. In this context it may be relevant to note that in the study by Wetzels and colleagues [33] following the expression of the basal epithelial keratins 14 and 17 in breast cancers, the tumors exhibiting basal cell characteristics were shown to express a hyperproliferation-related keratin (keratin 16) with much higher frequency.

These results also confirm the pathological classification of lobular and ductal carcinomas, and suggest that the lobular carcinomas develop from a cell that is more differentiated (i.e., more committed to the luminal cell lineage) and unable to express vimentin and keratin 14.

## 7. Intermediate filament expression in cell cultures and cell lines developed from normal and malignant mammary epithelial cells

To characterize the phenotype of cells cultured from a complex tissue like the mammary gland, it is important to have markers that will relate the phenotype of the cultures cells to that of the cells in vivo. Many parameters, such as growth factor receptors, are markedly altered by culture conditions. While this may also be true for the intermediate filament proteins in some cases, it is not always true. Thus, most estrogen-receptor-positive breast cancer cell lines (e.g., T47D, ZR75, MCF-7) and some estrogen-receptor-negative ones (e.g., BT20) express keratins 8, 18, and 19 homogeneously, and some also express keratin 7 [40,70]. Even after years of culture in different media, these cells do not express keratin 14, indicating that the profile of intermediate filament expression is a very stable feature of these malignant cells. The expression of vimentin in these lines remains controversial, but most laboratories report that estrogen-receptor-positive cell lines do not express vimentin [40,71].

It is important to remember that most breast cancer cell lines in general use were developed from metastatic cells in serous effusions. In this case, when the cells are growing in suspension, Raemakers and colleagues [72] have shown that vimentin expression is readily induced. Where intermediate filament proteins such as vimentin or keratin 14 are induced by culture, the

cell must be assumed to have the capacity to respond to the stimuli provided in culture by synthesizing these proteins. A cell that does this can, therefore, be clearly classified as of a different phenotype from one that does not. In fact there are several cell lines derived from breast cancers that express vimentin [73]. All of these are estrogen receptor negative and could represent less differentiated cells that grew out from the carcinoma.

One specific problem that exists in culturing epithelial cells from the normal breast is that cyclic AMP is required as a mitogenic stimulus to obtain growth. This reagent is known to induce squamous differentiation [74] and may induce the expression of keratin 14 and other keratins associated with stratified epithelia differentiation [5]. However, as noted earlier, a cell that is sufficiently committed to the secretory luminal cell lineage will not synthesize these keratins in culture, although a more uncommitted cell might. With all the problems associated with the use of intermediate filament expression to define cell phenotypes in culture, in combination with other markers of the luminal or basal lineage, the intermediate filament proteins are proving to be extremely useful.

### *7.1. Normal mammary epithelial cells*

**7.1.1. Milk epithelial cells.** The major sources of normal mammary epithelial cells are human milk [75,76] and reduction mammoplasty tissue [77,78]. These cells can be grown in monolayer culture on plastic [78] or embedded in collagen gels [79–81] using a modification of the technique developed by Emerman and Pitelka [82] for studying differentiation in mouse mammary epithelium.

As might be expected, all the cells that are shed into milk appear to be of the luminal epithelial cell type, since they express keratins 8 and 18 [83], and about 85% of colonies also express keratin 19 [39]. Interestingly, the colonies that are apparently keratin 19 negative are large, supporting the idea that the keratin-18-positive, 19-negative cells have a high proliferative potential. Although the luminal cells in vivo, particularly those in the TDLU, do not express keratin 14 or vimentin, a fraction of the cells cultured from milk do express these proteins. By introducing SV40 virus into individual cells [84] or by means of transfection, or infection with a retrovirus [54], we have isolated cell lines that can be classified according to the intermediate filament proteins they express (Table 3). The lines in Group 1 correspond, in the profile of intermediate filament that they express, to the luminal epithelial cell found in both the normal gland and most breast cancers. These cells do not respond to culture conditions by synthesizing keratin 14 or vimentin. We propose that the Group 3 lines correspond to the keratin-19-negative luminal cells that in culture can be induced to express keratin 14, and Group 2 represents a cell type defined by culture, i.e., expressing keratin 19 but still able to synthesize keratin 14 in culture. Although they synthesize keratin 14, neither the Group 2 nor the

*Table 3.* Subdivision of cell lines developed from milk epithelial cells on the basis of their intermediate filament protein expression pattern

| | Intermediate filaments expressed | | | |
|---|---|---|---|---|
| Cell lines | Keratin 7,8,18 | 19 | 14 | Vimentin |
| Group 1 | + | + | − | − |
| Group 2 | + | ± | + | − |
| Group 3 | + | − | + | − |
| Group 4 | + | − | − | + |

Group 3 lines synthesize vimentin. The Group 4 lines appear to be less differentiated and do express vimentin, but not keratin 14.

**7.1.2. Cultures from reduction mammoplasty tissue.** The mammary organoids that remain after collagenase digestion of reduction mammoplasty material and that form the starting material for tissue culture contain both basal and luminal cells. On plastic, two layers spread out from the organoid, and the upper layer shows a keratin profile (positive for keratins 8, 18, and 19) characteristic of luminal epithelial cells. They also express a mucin normally expressed by luminal cells. The lower layer, which is expected to arise from the basal cells, is epithelial, since the cells stain positively with antibodies to K14 and vimentin, but they are not stained with antibodies to keratins 18 and 19. Which of the cell populations proliferates depends on the media used. With the medium developed for growing the luminal cells from milk [85], the top layer proliferates, but these luminal cells, as with those found in milk, have a short in vitro lifespan. In the complex medium MCDB 170, developed by Hammond et al. [86] for the clonal growth of mammary epithelial cells, the cells emerging from the basal layer proliferate. However, most of these cells senesce after three to four passages [87], at which point a new cell type emerges that has a very extended lifespan. This cell expresses keratin 14 and vimentin (characteristic of basal cells), as well as keratins 7, 8, and 18 (characteristic of luminal cells); keratin 19, however, is not expressed by these cells [40]. In the light of the recent studies identifying keratin 14 and vimentin expression in association with highly proliferative lesions, it seems likely that the cell that is selected after passage 3–4 in the reduction mammoplasty cultures that shows features of both basal and luminal cells may have some of the properties of a putative stem cell. Cell lines have been developed by treatment with chemical carcinogens or by introduction of oncogenes, but their detailed phenotypes in terms of keratin expression have not been reported. However, immortalization has been reported to result in a decrease in keratin expression, particularly keratin 5 [70].

Other investigators have used different media for growing cells from reduction mammoplasty tissue. Using the complex MM medium, Dairkee

and colleagues [88] reported that proliferating cells express keratin 14 and, heterogeneously, the mucin characteristic of luminal epithelial cells. Curschellas et al. [89], using a similar medium, also found expression of intermediate filaments characteristic of both basal and luminal cells in vivo. In this case a high level of vimentin expression was noted in the outgrowths, as well as heterogeneous expression of the simple epithelial keratins. It appears that in using the MM medium some proliferation of both basal and luminal cells is observed for three to four passages, but more modification of the profile of intermediate filament expression is seen in this medium [40]. Extended growth of mammary epithelium can be obtained using the low calcium medium developed by Soule and McGrath [90], but characterization of the intermediate filament proteins expressed by the cultured cells has not been reported.

### *7.2. Cells cultured from breast cancers*

**7.2.1. Primary cancers.** It is notoriously difficult to culture demonstrably malignant cells from primary breast cancers. The most widely known cell line derived from this source is the estrogen-receptor-negative BT20. BT20 expresses keratins 8, 18, and 19, and has been found to be completely negative in its reaction with vimentin antibodies (J. Bartek, personal communication) [89]. Another cell line from a primary cancer, BT474, shows a similar profile of intermediate filament expression. Although there are several other lines reported in the literature to be derived from primary breast cancers, their intermediate filament expression has not been properly characterized.

The difficulty in obtaining cell lines from primary breast cancers indicates that the invasive cells in breast cancers are difficult to propagate. It is therefore crucial to use markers to examine the proliferating cells in short-term cultures. Antibodies to intermediate filament proteins, along with other markers, give some idea as to which cells are being propagated in different media. Using the medium MCDB 170 used for normal cells, the same cell type emerges as is seen in normal cell cultures, initially resembling basal cell, then acquiring stem cell properties. This cell usually has a shorter lifespan than the cells cultured from normal tissue. The cells are diploid, even when grown from aneuploid tumor [91], and it seems likely that the cells cultured in this medium do not come from the invasive compartment. The shorter in vitro lifespan could then be explained by the fact that only a fraction of the cells (nonmalignant or premalignant) are proliferating. We have used the low-calcium medium of Soule and McGrath [90] to culture cells from primary breast cancers and have found that most cells express both the simple epithelial keratins and keratin 14. In these cultures some squaming is apparent. Immortalization of individual cells with SV40TAg has led to the development of several cell lines from each of four tumors [92]. Only one of these (an argyophilic, mucoid tumor) induces tumors in the

nude mouse, and then only with low efficiency. This cell line expresses keratins 7, 8, 18, 19, and 14, but no vimentin. The cell lines from the other tumors (two infiltrating lobular and one infiltrating ductal) appear to have developed from the associated benign tissue or from the in situ component, as they neither grow in agar nor the nude mouse.

Our data again emphasize the problem of culturing malignant cells from primary breast cancers. In this context, it is perhaps relevant to point out that a malignant tumor is thought to be clonal in origin. In the case of breast cancers, the altered cell may correspond to the less differentiated phenotypes characterized variously as estrogen receptor negative and $EGFR^+$, keratin $14^+$, or vimentin positive. However, in most cases only a few divisions of transit cells may occur before converting to the final differentiated phenotype (keratin 8, 18, and 19 positive) seen in most invasive breast cancers. This differentiated phenotype must acquire division potential, since it constitutes the phenotype of most metastatic cells and cell lines developed from them. However, in the original tumor and certainly under culture conditions, proliferation of this phenotype is perhaps not high. If, then, the number of the highly proliferative cells is low and culture conditions favor their differentiation, it is obvious that it will be difficult to obtain cell lines from the malignant tumor cells.

**7.2.2. Metastatic tumors.** Not surprisingly, it appears to be somewhat easier to culture malignant breast epithelial cells from metastases. In our initial exploratory studies culturing cells from primary cancers and involved lymph nodes, we identified the putative malignant cell on morphological grounds and found these epithelial cells could be cultured for short periods from most involved lymph nodes but from only a fraction of primary cancers [93]. However, there are few reports of development of breast cancer cell lines from solid metastases, although some of those obtained from breast tissue may in fact be from secondary deposits within the breast. Most of the breast cancer cell lines that are widely used have, in fact, been developed from pleural effusions taken from late-stage cancer patients. Undoubtedly, it is much easier to obtain growth in culture of these malignant cells, where they may initially be stimulated to grow by growth factors produced themselves and/or by the mesothelial cells also found in the effusion. Also, although many of the cell lines show the intermediate filament protein profile of the differentiated luminal cell, the cells in the effusions have clearly acquired division potential. Perhaps because they have been chosen for positive estrogen-receptor content, the cell lines commonly in use (T47D, MCF-7, ZR75, CamaI) do, in fact, exhibit the phenotype of the differentiated luminal cell, as does SKBr3 (expression of keratins 8, 18, and 19). However, several cell lines, all estrogen receptor negative, have been reported to express vimentin and very low levels of keratins [73]. The cell line PC42 is of particular interest, as it appears to have stem-cell properties and contains subpopulations that are either vimentin positive (and keratin 19 negative) or

keratin 19 positive and vimentin negative [61]. Although a more detailed examination of the intermediate filaments in the less well-known cell lines is warranted, there is enough information to know that many of those derived from serous effusions do express vimentin. In earlier studies, Ramaekers and colleagues [72] were able to demonstrate coexpression of vimentin and keratins in metastatic carcinoma cells found in body fluids when vimentin expression was not detectable in solid primary or metastatic epithelial tumors. Thus, vimentin expression may be more common in cell lines derived from malignant effusions.

## 8. Concluding remarks

The application of monoclonal antibodies to intermediate filament proteins to the staining of tissue sections of normal, benign, and malignant mammary gland has confirmed the classification of cell and tumor types based on pathological and morphological criteria, and has led to subclassifications based on the profile of intermediate filament expression. There is a clear distinction between the majority of basal and luminal cells, based on the expression of keratins 5, 14, and 17 in the basal cells, and of keratins 7, 8, 18, and 19 by the luminal cells.

It is of interest that all the subsets of cells seen in either the basal or luminal layer, which differ from the general classification in their profile of intermediate filament protein expression, are to be found in the small ducts and TDLU, where most proliferative activity is to be found in the adult and where the development of breast cancer is thought to occur. This could suggest that these subsets do indeed define differentiation phenotypes that are transit cells in the differentiation-orientated proliferation of a stem cell, or stem cells leading to the functioning luminal or basal epithelial cell. In the human, however, it is difficult to obtain direct evidence for such an assumption, unless the whole differentiation pathway can be reproduced in vitro. One experimental system with great potential is to examine in detail the profile of intermediate filament expression in the structures that develop in the nude mouse when reduction mammoplasty organoids are implanted. Studies in this system showed that the keratin-19-negative luminal cell compartment increased in these structures [94], but other epithelial subsets were not examined.

Certain clues regarding cell phenotypes are beginning to appear from the detailed immunohistochemical analysis of breast tumors. In benign tumors there seems to be an increase in some of the subsets of cells defined by a different profile of intermediate filament protein expression. Since this increase is seen in association with increased abnormal (and probably polyclonal) proliferation of the mammary epithelial cells, it suggests that some of these subsets may form separate proliferative compartments.

An examination of IFP expression in malignancy shows that around 90%

of invasive cancers resemble the differentiated luminal epithelial cell of the normal breast in the profile of intermediate filament proteins that are expressed. However, the association of a bad prognosis of the small percentage of tumors that express the 'basal' IFPs, keratin 14 and vimentin, is of great interest. This subset of tumors also show a high expression of the EGF receptor, which is also found predominantly in basal cells (R. Nicholson, personal communication) and an inverse correlation with the presence of estrogen receptor, which is only seen in luminal cells in the normal breast. The high growth fraction and expression of hyperproliferative keratins in the tumors expressing basal markers supports the idea that a less differentiated phenotype, normally found in the basal layer, which may be a precursor of the luminal cell, may be a target cell for the initiation of malignancy, at least in this subset of tumors.

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# 18. Casein gene expression: from transfection to transgenics

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## 1. Introduction

Milk-protein gene expression during pregnancy and lactation signals the terminal differentiation of the developing mammary gland. Hence, the synthesis and secretion of milk proteins serve as molecular markers with which to assay the action of both peptide and steroid hormones, as well as the cell-substratum interactions required to achieve lactation. For many years researchers were limited to descriptive analysis of milk-protein gene structure and expression using primary mammary epithelial cell lines and explant cultures. Now, with the advent of modern gene-transfer technology, the molecular mechanisms regulating milk-protein gene expression can be more thoroughly studied using transgenic mice and transfected mammary epithelial cell lines. However, both these systems have their limitations. Derived from midpregnant mice, the currently available mammary cell lines do not phenotypically represent the mammary epithelial cell during lactation. These cells do not express the complete spectrum of milk proteins, and the level of expression of many of these genes, even under the best culture conditions, is markedly less than that observed during lactation. However, using pooled transfectants does permit the simultaneous analysis of multiple recombinant plasmid constructs. Such studies, certainly less expensive than those in transgenic mice, also circumvent the often deleterious effects associated with random transgene integration. On the other hand, transgenic mice facilitate a comprehensive evaluation of milk-protein gene expression within the context of a developing animal. Explant cultures can also be derived from individual lines of transgenic mice and can be used to study the response of a transgene to individual hormones. In addition, lines of transgenic mice can be established using transgenes designed to direct oncogene expression to the mammary gland from which immortalized cell lines may be isolated. Such cell lines may represent different stages of mammary gland development and, therefore, can be used to investigate how oncogene expression results in mammary carcinogenesis. From this brief introduction it is evident that both cell culture and transgenic systems should

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

be employed in order to effectively study mammary gene expression at the molecular level.

This chapter will focus primarily on our attempts to understand the molecular mechanisms that regulate β-casein gene expression. Although our laboratory has also been characterizing whey acidic protein gene expression, this topic is addressed in Chapter 19.

## 2. Milk-protein genes

In the late 1970s our laboratory initiated a project to isolate cDNA clones for several of the major rat milk proteins [1]. Once isolated and mapped, their complete nucleic acid sequences were determined. These cDNA clones were subsequently used to probe gene libraries and to isolate several of the genes encoding the caseins and whey proteins [2]. The rat whey acidic protein (WAP) and β-casein genes were the first milk-protein genes to be completely characterized [3,4]. The primary structures for WAP, β-casein, and several other milk-protein genes have now been determined in a number of additional mammalian species [reviewed in 5].

### *2.1. Structure and conserved promoter sequences*

Once the primary DNA sequence had been determined for a number of the calcium-sensitive casein genes, these sequences were compared. Several regions of similarity were identified in the regions immediately upstream of the structural genes. These sequences were, therefore, implicated as potential regulatory domains responsible for the mammary-specific and hormonally regulated expression of these genes [5–7]. The κ-casein gene promoter does not appear to contain any of the sequence motifs conserved between the calcium-sensitive caseins [8]. However, several of these motifs have been identified in a variety of whey-protein gene promoters [5,7]. For example, some similarity exists between the so-called 'milk box' consensus sequence motif, located at −150 bp in the casein gene promoters, and promoter regions of α-lactalbumin, WAP [reviewed in 5], and the mouse mammary tumor virus long terminal repeat (MMTV-LTR) (Figure 1A). Likewise, the motif found at approximately −90 bp 5′ to the start site of casein gene transcription is similar to a sequence found at −270 bp in the sheep β-lactoglobulin gene promoter (Figure 1B).

The promoter sequences found immediately 5′ to the mRNA start site of the milk protein genes, though essential for transcription, are not by themselves sufficient to elicit the appropriate regulation of these genes. Other more distal elements, such as locus control regions (LCRs) and enhancers, as well as intragenic sequences important for RNA processing and mRNA stability, are required to achieve the levels of expression observed for the endogenous milk-protein genes. Hence the identification of

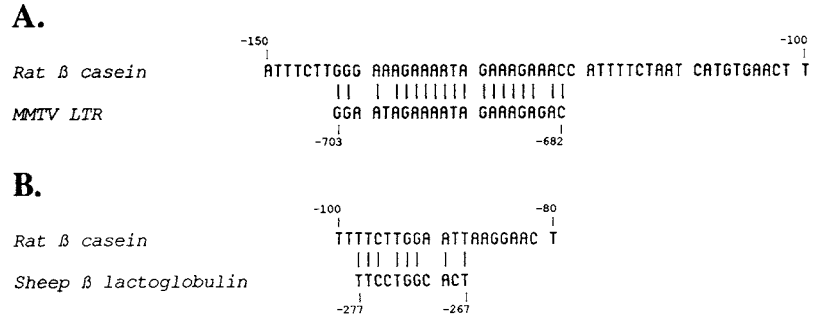



*Figure 1.* Sequence comparison between the promoter regions of genes expressed preferentially in the mammary gland. A: Similarities between the rat β-casein and mouse mammary tumor virus long terminal repeat promoter regions. B: Similarities between the rat β-casein and the sheep β-lactoglobulin promoter regions.

milk-protein gene promoters by computer-aided sequence alignment is only the first step in identifying potential sites of interaction with tissue-specific, developmentally and hormonally regulated *trans*-acting factors. The importance of these sites must, therefore, be established through functional bioassays. Usually, recombinant gene constructs are designed with the putative regulatory element placed in front of a minimal promoter fused to a reporter gene such as bacterial β-galactosidase (lacZ) or chloramphenicol acetyltransferase (CAT). The functionality of the element can then assayed either by transfection into mammary cell lines capable of maintaining endogenous milk-protein gene expression or by microinjection into zygotes to generate lines of transgenic animals.

### *2.2. Transfection into mammary epithelial cells*

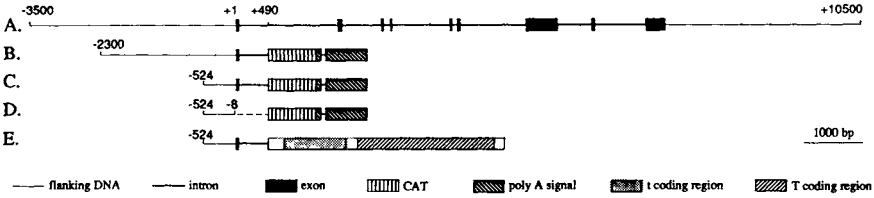
Numerous tumorigenic mammary epithelial cell lines have been isolated and characterized, yet few epithelial cell lines exist that display the specific morphological and functional differentiation unique to the lactating mammary gland. The first example of a cell line to exhibit several unique functions of a normal mammary gland was the COMMA-D cell line [9]. Derived from mammary glands of midpregnant mice, COMMA-D will express β-casein mRNA in response to lactogenic hormones, but only when cultured on an appropriate substratum. Unfortunately, only a small proportion of the cells expressed casein, as COMMA-D is composed of several different mammary epithelial cell types [10,11]. Additionally, the level of certain milk-protein mRNAs and *trans*-acting factors in COMMA-D do not reflect those observed in the mammary gland at lactation.

Initially, transfection experiments designed to study the regulation of entire milk-protein genes or promoter-reporter gene constructs in COMMA-D cells were unsuccessful. Recent transfection studies, using either a clonal derivative of the COMMA-D cells, designated HC11 [12], or an enriched

subpopulation designated CID9, have provided important insights into the mechanisms of hormonal and cell substratum regulation of milk-protein gene expression. The success of these transfection experiments relates in part to the use of enriched cell populations and to the analysis of stably, as opposed to transiently, transfected cells. Unlike the COMMA-D cells, the derivative HC11 cells no longer respond to substrata, and the induction of β-casein gene expression in HC11 does not require insulin (I) in the presence of prolactin (M) and glucocorticoids (F) [13]. The CID9 cells also exhibit hormonal and matrix-dependent β-casein and WAP gene expression, although the mRNA levels for these genes are lower, 10- to $10^4$-fold, respectively, than their maximal levels observed during lactation [14] (Dale and Schmidhauser, unpublished data). Recently, a transient transfection assay has been reported using β-casein-CAT reporter gene constructs in primary murine mammary epithelial cells [15]; however, a large variability in the response of primary cultures was observed in independent transfection experiments.

The use of β-casein reporter gene constructs with progressive deletions of 5′ flanking DNA has delimited the regions required to elicit hormonal and cell-substratum regulation in the rat and bovine gene promoters, respectively [14,16]. Some of these results are summarized in Figure 2. Further 3′ deletions in which the β-casein promoter fragments are linked to a heterologous promoter have also been used to define the 3′ boundary for hormonal responsiveness (B. Groner and W. Doppler, personal communication). In this fashion, the hormone-response elements have been localized to an approximately 200-bp region of the β-casein promoter, between −80 and −280 bp 5′ to the transcriptional start site. Similarly, the substratum-response element(s) have been localized within the first 1790 bp of the bovine β-casein promoter. Distinct prolactin and glucocorticoid response elements have not been identified using these constructs. The effect of glucocorticoids on casein gene promoter activity appears to be mediated indirectly via an uncharacterized prolactin signaling pathway (see below). So far there is no evidence for a direct interaction of glucocorticoid receptor with response elements in the β-casein promoter [16].

Despite the recent cloning of the prolactin receptor [17], very little is known about the mechanism of signal transduction involved in the regulation of milk-protein gene expression by prolactin. The prolactin receptor is a member of a larger family of cytokine receptors, which has been designated the hematopoietin receptor superfamily [18]. The extracellular, ligand-binding domains of these receptors display significant homologies, but they have quite different cytoplasmic domains. Several forms of the prolactin receptor exist in the mammary gland that differ in their intracellular domains as a result of alternative mRNA splicing. To localize the regions of the prolactin receptor important for the induction of milk-protein gene expression, a cotransfection assay has been developed by Leseur et al.




| | Transfection Studies[a] | | Transgenic Studies | | | |
|---|---|---|---|---|---|---|
| | | | Mammary Gland[b] | | | |
| Transgene | HC11 | NIH 3T3 | Expression | Development[c] | Explant[d] | Thymus/Spleen |
| A. Rat ß-casein, 14 kb | ND[e] | ND | 1 [20][f] | 10(MP->L)[20] | >25[31] | -[g] |
| B. -2300/+490 CAT | 20-40[13] | 0 | 17.95[h][26] | 3-19(V->L)[26] | 1.5[31] | 3.15[h][26] |
| C. -524/+490 CAT | 11-22[13] | 0 | 69.35[i][26] | 70(V->L)[26] | 2-43[1] | <0.01[26] |
| D. -524/-8 CAT | 7[j] | 0[j] | 0[j] | ND | ND | 0[j] |
| E. -524/+490 SV40 T/t ag | 5-10[k] | ND | see text | ND | ND | Tumors |

[f]references in [ ]
[a]fold induction observed for IMF versus I alone in stably transfected cells[13]
[b]biopsied from female mice at lactation
[c]fold-induction observed between virgin (V) or midpregnant (MP), and lactating (L) mammary gland as indicated
[d]fold-induction observed for IMF versus IF in explant cultures derived from transgenic mice at lactation[31]
[e]not determined
[f]expression of transgene as % of endogenous mouse ß-casein mRNA as determined by RNase protection
[g]not detected by RNase protection [20]; Detectable by RT-PCR (Greenberg and Rosen, unpublished data)
[h]CAT activity (% conversion/100ug protein; 8-10 hour assay) for line 5494 [26]
[i]CAT activity in line 5316 (see comment h)
[j]Greenberg and Rosen, unpublished data
[k]Wolfe and Rosen, unpublished data

*Figure 2.* Expression of rat β-casein (RβC) constructs in gene transfer experiments. The top panel depicts the constructs as discussed in the text. A, rat β-casein 14-kb transgene; B, −2300/+490 RβC-CAT transgene; C, −524/+490 RβC-CAT transgene; D, −524/−8 RβC-CAT transgene; E, −524/+490 RβC-SV40 T/t antigen transgene. The bottom panel summarizes transfection and transgenic studies with the constructs depicted above.

[19]. In this assay, expression vectors encoding the different forms of the prolactin receptor, as well as a milk-protein gene promoter-CAT reporter gene construct, are introduced into CHO cells. In the presence of prolactin, the long form of the receptor, but not the short form, resulted in a 17-fold induction of CAT activity. The success of these experiments is surprising, since CHO cells would not have been expected to express milk-protein promoter-CAT constructs, presumably due to the lack of transcription factors specific to mammary epithelial cells. However, the constructs presumably exhibited sufficient basal promoter activity to detect an effect mediated through a prolactin response element. Using this approach, in conjuction with more detailed mutagenesis analysis of the milk-protein gene promoters, it should be possible to delimit both the critical regions of the receptor involved in signal transduction at the level of the cell membrane and the sequences in the milk-protein gene promoters interacting with nuclear transcription factors. An additional challenge will then be to link these two processes to elucidate the precise prolactin signal transduction pathway.

*2.3. Expression in transgenic animals*

Transgenic mice make it is possible to analyze the developmental, tissue-specific, and hormonal regulation of milk-protein genes within the context of the developing mammary gland. In many cases the level of expression of the transgene may be directly compared against that of its endogenous counterpart. Studies in transgenic mice were initiated in our laboratory in 1984, due to the lack of suitable mammary epithelial cell lines that mimicked milk-protein gene expression in the lactating gland. Independent groups have to date generated transgenic mice that harbor the entire rat β-casein gene [20], the rat and mouse WAP genes [21,22], the sheep β-lactoglobulin gene [23], and the bovine and guinea pig α-lactalbumin genes [24,25]. In all cases the transgenes are expressed predominantly in the mammary gland during lactation. Interestingly, rat β-casein (RβC)-driven CAT-fusion gene constructs were found to be expressed in the thymus, but were regulated differently than in the mammary gland [26] (Figure 2). RβC-CAT activity decreased in the thymus during pregnancy and lactation, in contrast to the increasing levels of activity observed in the mammary gland. That thymic expression was not ectopic, or due to an unusual tissue specificity generated by the architecture of the RβC-CAT reporter gene, is explained by the observation that several members of the casein gene family are endogenously expressed in cytotoxic T lymphocytes [27]. Similar results also have been observed using other reporter genes (see below). In addition, in situ hybridization studies have recently identified guinea pig α-lactalbumin mRNA transcripts and endogenous β-casein mRNA transcripts in the undifferentiated basal layer cells of sebaceous glands of transgenic mice during lactation [24]. These interesting results notwithstanding, expression of the endogenous milk-protein genes and the β-casein and whey protein transgenes is found predominantly in the mammary gland.

There is now good evidence that the sequences important for mammary-specific gene expression and the proteins that interact with these sequences are well conserved. This conclusion is based on the ability of milk-protein genes from one mammalian species to be efficiently expressed in another mammalian species. For example, the sheep β-lactoglobulin gene is efficiently expressed in transgenic mice [23], and the mouse WAP gene is expressed at high levels in transgenic pigs [28]. However, not all transgenes have been observed to be expressed with the same efficiency as their endogenous counterparts. Although the individual whey protein genes, with only 750–915 bp of 5′ flanking DNA, are expressed quite efficiently in transgenic mice [21,25,29], the expression of a rat β-casein construct containing 3.5 kb of 5′ and 3.0 kb of 3′ flanking genomic DNA was inefficient in transgenic mice [20]. These observations suggest the β-casein transgenes were missing distal positive regulatory sequences.

Although initial attempts to study the hormonal regulation of β-casein promoter-CAT constructs were unsuccessful in transiently transfected

COMMA-D cells [30], the same −524/+487 promoter fragment was able to direct CAT expression to the mammary gland in transgenic mice in an appropriately regulated fashion during mammary development [31]. Explant cultures derived from these mice displayed a two- to four-fold induction of CAT activity in response to prolactin and glucocorticoids [26]. However, when the same construct was studied in stably transfected HC11 cells, an 11- to 22-fold induction of CAT activity was observed for IFM vs. I alone [13]. No induction of CAT activity was evident in stably transfected NIH 3T3 cells (Figure 2). To better define the regulatory elements mediating this hormonal effect, a −524/−8 β-casein promoter-CAT construct was introduced into both NIH 3T3 and HC11 cells. Deletion of the β-casein first exon and part of the first intron resulted in a decreased basal activity (i.e., I alone) in HC11 cells and the loss of detectable activity in NIH 3T3 cells in both I- and IFM-treated cells. Thus, the DNA sequences responsible for both mammary epithelial-specific gene expression and lactogenic hormone induction appear to reside within the approximately 500 bp of 5′ flanking DNA in the β-casein gene promoter. To confirm this result, the same construct was used to generate eight independent lines of transgenic mice. Surprisingly, there was no detectable CAT activity observed in the mammary gland of 10-day lactating mice (Greenberg and Rosen, unpublished observations). The apparent discrepancy between the data obtained from transgenic mice and transfected cells may be related to an inappropriate intron architecture in the construct, as introns are known to be required to enhance the expression of transgenes in mice [32]. Thus, it will probably be necessary either to insert a heterologous intron 5′ to the CAT gene or to use a genomic reporter gene in order to further test these 'promoter-only' β-casein constructs in transgenic mice.

The casein genes are part of a gene cluster located on mouse chromosome 5 [33] and bovine chromosome 6 [34]. Pulse field gel electrophoresis has demonstrated their physical linkage in the order αS1-β-αS2-κ within 200 kb of DNA [34, 35]. Thus, the β-casein gene resides in the middle of a large gene locus that may be under the influence of distal locus activating regions (LARs) that were not included in the constructs used in the transfection or transgenic studies. Necessary for chromatin-organizing events during development, LARs permit the subsequent expression of genes within a locus and are characterized by their hypersensitivity to DNase I digestion. These regulatory sequences have also been designated dominant control regions, because they conferred copy-number-dependent and position-independent transgene expression in mice. The α- and β-globin gene clusters, by analogy, have remote positive regulatory sequences present 30–50 kb upstream that are required for efficient globin gene expression in transgenic mice [36,37]. The β-globin LAR contains four very strong DNAse I hypersensitive regions, two of which have been shown to interact with a variety of tissue-specific and ubiquitous *trans*-acting factors in a cooperative manner [37,38]. These two sites were sufficient to direct the

efficient expression of a β-globin transgene [39]. Although β-globin LARs can activate both homologous and heterologous promoters in a tissue-specific manner [40], they require β-globin introns to elicit efficient transgene expression [39]. In addition, LARs may not elicit copy-number-dependent expression should some transcriptional factors become limiting with a high transgene copy number [36]. Finally, it should be mentioned that the effect of LARs is only observed in gene constructs tested in transgenic mice and stably transfected cell lines, but not in transiently transfected cells, suggesting that higher order chromatin structure is a critical determinant of LAR effects on gene expression.

As discussed above, the WAP gene is expressed at high levels in transgenic mice, while the entire β-casein gene is expressed rather poorly. This difference may be attributed to the lack of remote positive regulatory sequences in the 14-kb β-casein gene construct, which may reside within the 4.3-kb rat WAP gene construct. Since a variety of constructs in which the WAP promoter contained 2.6 kb of 5′ flanking DNA have failed to express efficiently [41], our laboratory turned to the task of identifying putative enhancer elements within the 4.3-kb rat WAP construct used in our previous studies [21]. By analogy with the β-globin genes, we first focused on the 1.3 kb of 3′ flanking DNA in the initial WAP gene construct [41b]. Accordingly, deletions were generated and one construct, which contained only approximately 70 bp of 3′ flanking DNA, was used to generate independent lines of transgenic mice. Of nine lines carrying the transgene, eight expressed at high levels equivalent to or greater than the level observed in rat mammary gland at 10 days of lactation. In the lactating rat, WAP mRNA comprises more than 10% of the total mRNA [42]. The uniformly high levels of transgene expression were less variable than observed with the larger 4.3-kb rat and 7.2-kb mouse transgenes [21,22]. Thus, it appears that the elements required for efficient WAP expression lie within approximately 3.0 kb of DNA. However, an alternative explanation is that a negative regulatory element has been deleted in the shorter constructs, as negative regulation appears to play a role in both the developmental and cell-substratum regulation of WAP gene expression [21,43] (T. Dale and J. Rosen, unpublished data).

Site-independent expression in transgenic mice has been reported for a 5.5-kb T-cell-specific CD2 transgene and a 4.5-kb chicken $\beta^A$-globin transgene [44,45]. These studies demonstrate that position independence can be mediated by sequences within 2 kb from a gene, and, in some cases, the sequences need not define a gene cluster boundary. However, should certain *trans*-acting factors become limiting, copy-number-dependent expression would no longer be observed. Unexpectedly, position-independent expression of the rat WAP gene has been demonstrated to require the presence of 91 bp of conserved WAP 3′ untranslated sequences [41b].

### 2.4. *Regulation by* trans-*acting factors*

Once the general region of the β-casein gene promoter required for hormonal induction and mammary epithelial cell-specific expression has been localized within a few hundred base pairs, it is possible to investigate how nuclear proteins interact with specific DNA sequences. A series of overlapping oligonucleotides have now been synthesized that span the region approximately −34 to −300 bp 5′ to the start site of β-casein gene transcription. These oligonucleotides are being used with nuclear and/or whole cell extracts prepared from either cell lines or tissues in electrophoretic mobility shift assays and DNA footprinting experiments to determine if specific protein-DNA complexes are formed. Several specific complexes have been observed from both liver and mammary nuclear extracts. One slowly migrating complex was observed that appears to be mammary specific. Bernd Groner and his colleagues [46] have also identified a mammary-specific nuclear factor(s) that interacts with the conserved sequence at −90 shown in Figure 1. Despite the preferential expression of β-casein-CAT constructs in HC11 cells, it has not been possible, however, to detect this factor(s) in unfractionated nuclear extracts. The HC11 cells were derived from a midpregnant mammary gland, and it now appears that expression of this factor(s) is regulated during mammary development [46b]. At present, it still remains to be established by functional assays whether this factor(s) and its target sequence are capable of eliciting mammary-specific expression. Further purification of the factor(s) and ultimately gene cloning will be required to validate this hypothesis.

It has been suggested that hormonal regulation of β-casein gene expression might act via prolactin-mediated relief of transcriptional repression mediated through sequences within the 'milk box' region [46]. This operating hypothesis was based on the analysis of mutations within the β-casein promoter known to prevent the interaction of factor(s) with this region. To test this hypothesis in vitro, we placed milk-box oligonucleotides at position −105 relative to a thymidine kinase constitutive promoter-CAT reporter gene fusion (designated pBL2CAT [47]). Thus, the spacing of the milk-box elements would be relatively similar to that observed in the β-casein promoter. Surprisingly, the addition of one or three copies of this element to pBL2CAT did not result in a repression of CAT activity, but caused increased CAT activity in NIH 3T3 and HC11 cells. The addition of prolactin and glucocorticoids to the transfected cells yielded no further increase in CAT activity. These experiments, therefore, suggest that should prolactin-mediated relief from the transcriptional repression mechanism be responsible for hormonal regulation of β-casein gene transcription, then this only occurs within the context of the wild-type promoter. Furthermore, hormonal responsiveness appears to be organized through a complex interaction of several factors and is clearly dependent on the promoter context.

## 3. Targeting oncogenes to the mammary gland

The ability to direct oncogene expression to a specific tissue or set of tissues in transgenic mice facilitates the characterization of the molecular mechanisms governing transformation of differentiating tissue within the context of an intact developing animal. Such investigations are important to cancer research because they account for the physiological factors, such as immune surveillance and humoral and autocrine responses, that play central roles in the manifestation of malignancies in vivo. To investigate how the timing and level of oncogene expression in a defined cell type can affect normal mammary development, various groups have generated lines of transgenic mice with a myriad of oncogenes fused to regulatory elements that direct transcription to mammary tissue.

### *3.1. Mammary-specific promoters*

Only a handful of promoter/enhancer elements, viral or cellular in origin, are known to function preferentially in mammary tissue, and a considerable effort has been made to characterize their various molecular control mechanisms. These studies have shown that each promoter has a unique pattern of expression with respect to timing and to tissue and cell preference. Therefore, research groups often use different promoter- and enhancer-based constructs to study mammary gene expression. This is a particularly useful approach for studying the effects of oncogene expression in the changing environment of the mammary gland during the life cycle of the animal. Another advantage of oncogene targetting is the potential to establish immortalized clonal cell lines. Such lines, each essentially fixed in time with respect to various stage of mammary gland development, can be used to identify, isolate, and characterize genes associated with the development and maintenance of the neoplastic state. Analysis of these genes and their associated protein products can help identify the critical regulatory pathways that become perturbed in the development of cancer.

One of the most studied mammary restricted promoters is contained within the mouse mammary tumor virus long terminal repeat (MMTV-LTR). Essential for proviral expression, the LTR is also responsible for the activation of the *int* genes, so named for their proximity to the integration site of the provirus. The gene originally identified as *int-1* is now termed *wnt-1*, as it is the murine homology of the *Drosophila* segment polarity gene, wingless. *Wnt-1* has been implicated as playing a role in the developing neural crest and brain. The *int-2* gene, which is not related to *int-1*, is a member of the basic fibroblast growth factor family and may be involved in the development of mammary epithelial hyperplasia.

The MMTV-LTR has been widely used to drive heterologous gene expression in both transfection and transgenic systems. Although proviral MMTV is expressed predominantly in the lactating mammary gland, LTR-

based transgene constructs have been found to express in mammary gland, salivary gland, lung, kidney, thymus, spleen, and testes of transgenic mice [48–52]. One of the problems encountered in the generation of transgenic mice with LTR-driven constructs is that expression in the testis often results in sterile males. Male sterility can make it difficult to propagate lines with important phenotypes but can be overcome using some rather clever schemes. Using a 'binary transgenic system' [53] the *int*-2 cDNA was targeted to the mammary gland but was only expressed when independently generated 'target' and 'transactivator' lines were bred to generate F1 animals. The transactivator lines carried LTR-driven transgenes to express the yeast GAL4 protein transcription factor in mammary tissue. The target line carried a yeast GAL4 upstream activation sequence (UAS)-*int-2* fusion gene. Therefore, only in the F1 animal will the GAL4 protein interact through the UAS to activate transcription of the *int-2* oncogene expression system in the mammary gland. This paradigm can be applied to almost any gene and any tissue, provided suitable regulatory elements exist to express the transactivator protein in the tissue of interest.

Regulatory sequences derived from the genes encoding the major milk-protein β-casein and the major whey protein of rodents, whey acidic protein, have also been used successfully to direct heterologous gene expression to the mammary gland of transgenic mice [for a review see 41]. Like MMTV-LTR, the endogenous whey and casein genes express almost exclusively in the lactating mammary gland. However, their level of expression during pregnancy and lactation differ remarkably with respect to the timing and response to lactogenic stimuli. For example, initial studies on the regulation of mammary-specific gene expression during growth and differentiation demonstrated that β-casein expression occurred in the virgin mammary gland, increased markedly at day 10 of pregnancy, and by lactation β-casein mRNA had increased 250-fold to account for 20% of mRNA in the mammary gland at day 8 of lactation. Basal WAP transcription, on the other hand, was found to be very low in the developing mammary gland, yet is induced several thousandfold beginning at day 15 of pregnancy. These examples serve to illustrate the range of spatial and temporal expression patterns of the MMTV-LTR and milk-protein promoters that can be exploited in transgenic studies.

The MMTV-LTR promoter/enhancer and WAP promoter have both been used to express the activated human Ha-*ras* [54–57] and c-*myc* [55,57] oncogenes in transgenic mice. The MMTV/Ha-*ras* transgene was found to be expressed in many tissues, and this expression correlated with a number of distinct phenotypes. The WAP/Ha-*ras* transgene, on the other hand, expressed predominantly in the mammary gland and the brain of lactating females. Interestingly in both cases, mammary tumors only arose after long latency periods, despite high-level expression of Ha-*ras*. Since one genetic 'hit' is probably insufficient to establish a malignant transformation, transgenic experiments have been designed to determine the potential for dif-

ferent oncogenes to cooperate. When the MMTV/Ha-*ras* and MMTV/*myc* transgenes were coexpressed, the mice had a higher incidence of tumors than the parental lines expressing only the individual transgenes [55]. However, coexpression of the WAP/Ha-*ras* and WAP/c-*myc* transgenes caused only preneoplastic lesions and impaired development and organization of the mammary epithelium [57]. These observations illustrate how the unique spatial and temporal expressivity of the promoter can lead to the elaboration of distinct phenotypes in a particular tissue.

The response mediated by the expression of a particular oncogene is often cell-type specific, and the SV40 early genes have been associated with the development of a number of neoplastic phenotypes. It was hoped that the transforming potential of the SV40 large T and small t antigens (T-ag; t-ag) could be used to establish transformed cell lines that express milk and whey proteins in response to lactogenic stimuli. Therefore, we have attempted to use a rat β-casein promoter-SV40 early gene construct to establish clonal cell lines from mouse mammary epithelial cells, since the mammary gland was found to be refractory to neoplastic transformation by MMTV-LTR-driven T/t-ag constructs in transgenic mice [51,52].

### *3.2. β-casein-directed oncogenes in transfection studies*

Constructs were designed to express the SV40 early genes with a minimal 1-kb promoter fragment of the rat β-casein gene (0.5 kb of 5′ flanking DNA, noncoding exon 1 and 0.5 kb of intron A) that had been shown in transgenic mice to direct heterologous gene expression to the mammary gland [26] (Figure 2). Transfection experiments were used to characterize the effects of early SV40 gene expression specifically on mammary epithelial cells in culture. These studies were performed in the COMMA-D-derived, clonal mouse mammary epithelial cell line designated HC11. The HC11 cells are unique in their ability to differentiate and synthesize β-casein in response to lactogenic hormones when grown to confluence on a plastic culture dish [12,13].

When the T/t-ag transformed HC11 cells were treated with insulin, hydrocortisone, and prolactin to induce differentiation and β-casein expression, a rapid yet transient increase in T-ag expression was observed at the mRNA and protein levels. The transient nature of this expression is believed to result from transcriptional inhibition arising through the interaction of T-ag protein with an SV40 domain present on the construct. Expression of T-ag caused arrested HC11 cells to reenter the S phase, and to accumulate histone H4 RNA. Despite their altered growth characteristics, the cells did not display a characteristically transformed phenotype, as they failed to grow in soft agar, nor did they form tumors when injected into the cleared mammary fat pads of syngeneic mice. However, when transfected cells were cultured for 24 hours with prolactin and injected subcutaneously into four nude mice, anaplastic carcinomas arose after a 12-week latency

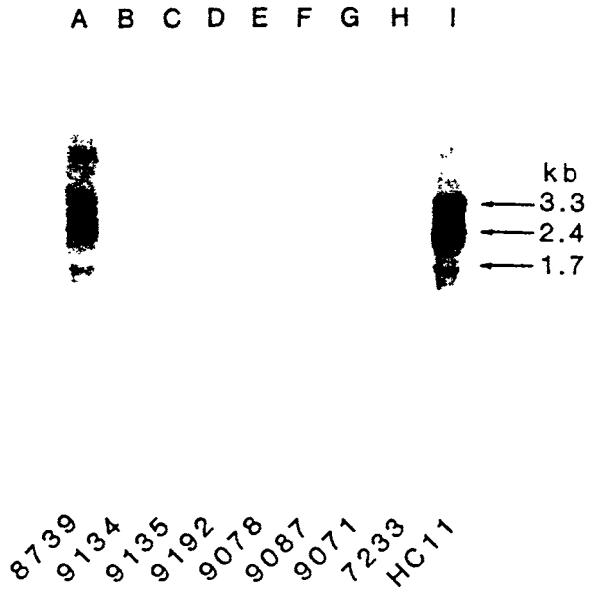



*Figure 3.* Expression of the −524/+490 rat β-casein-SV40 T/t antigen construct in transgenic mice. RNA isolated from the fourth mammary gland biopsied from female founder mice at day 10 lactation was separated on formaldehyde gels and probed with an SV40 T antigen-specific probe. The probe recognizes three species of mRNA from the 8739 mouse and the HC11 transfected cells.

period. These carcinomas were not observed in control experiments using HC11 cell transfected with the $pSV_2neo$ plasmid. Since HC11 cells transfected with a variety of oncogenes (Ha-*ras*, *neu*T, c-*erb*B2, TGF-α [58]) have been shown to be tumorigenic with a latency of less than 3 weeks, the T/t-ag constructs, therefore, were not capable of transforming HC11 cells in vitro, yet converted the cells to full malignancy in vivo. In fact, recent experiments have demonstrated that wild-type p53 protein (p53wt) can block the initiation and maintenance of SV40 transformation in precrisis as well as established mouse fibroblast cell lines [59]. In pulse-labeling studies, relatively high levels of apparently mutant and wild-type p53 as determined by their reactivities with specific monoclonal antibodies were detected in the T-ag transfected HC11 cells, which could then interact with T-ag, thereby preventing transformation of the HC11 cells in vitro [59b].

### *3.3. β-casein-directed oncogenes in transgenic studies*

To determine the consequences of T-ag expression during mammary gland growth and differentiation, independent lines of transgenic mice were generated with the β-casein T/t-ag construct. Between 4 and 12 months of

age, both the male and female mice developed a number of malignant lymphomas of the generalized histiocytic type in the spleen and uterus. Although this type of tumor has been observed infrequently in older mice, our data suggest these lymphomas resulted as a consequence of transgene expression, as most mice died by 5 months of age. One founder animal died from a bone tumor at 14 months of age. At necropsy, lymphomas were found to be multicentric, with accompanying involvement of bone, muscle, and peripheral lymph nodes. In the four lines examined in detail, there was involvement of the spleen (4/4), liver (3/4), large intestine (3/4), and uterus (2/4). Line 8739, which expressed high levels of T-ag mRNA in the mammary gland (Figure 3) ultimately died of an ovarian tumor. It was difficult to breed some lines, possibly due to the uterine or ovarian involvement, although we were able to carry most lines until the F2 generation. Because no mammary tumors were observed in the female mice, even after multiple pregnancies, the transgenic studies and the tranfection experiments strongly indicate that, compared with other tissues, the mammary gland is not very sensitive to transformation by the SV40 T-ag genes. Similar results have been observed with the MMTV/T-ag construct [52]. Despite the inability of the the β-casein/T-ag construct to cause mammary tumors, a number of cell lines are now being established from the other malignant lymphomas obtained in the transgenic studies. These cell lines will be useful in characterizing the molecular events that lead to their transformation and, hopefully, the cell type within which the tumor originated.

We have also used transgenic mice to investigate the consequence of expressing the *neu* oncogene in the developing mammary gland. The *neu* oncogene, originally isolated from neuro/glioblastomas obtained from ethylnitrosourea-treated rats, encodes a 185-kD epidermal growth factor receptor-related protein [60,61]. Although implicated in the normal development of epithelial tissues [62], amplification of the human *neu* homolog, *erb*-B-2, has been reported in a number of human breast and ovarian cancers [63,64]. To target *neu* to the mammary glands in transgenic mice, the minimal −524/+490 rat β-casein fragment, as described above, was fused to cDNAs encoding either the wild-type (*neuN*) or the activated (*neuT*) oncogenes. The activated *neuT* carries a point mutation (valine to glutamic acid) at position 664 in the transmembrane coding domain that reportedly increases the intrinsic tyrosine kinase activity of the receptor-related protein [65,66]. These constructs were used to generate seven independent lines of transgenic mice with the *neuT* construct and three lines with the *neuN* construct. However, after approximately 1 year there were no palpable tumors detected in mice of either sex in any of the ten lines. Only low levels of *neu* mRNA were detected by reverse transcriptase-mediated PCR in the mammary tissue biopsied from female mice at day 10 of lactation. These results are in contrast to those using the MMTV-LTR to drive expression of c-*neu* in transgenic mice. In the studies by Muller et al. [67],

mice expressing a MMTV/activated-c-*neu* transgene developed mammary adenocarcinomas involving the entire epithelium in each gland, and the activated *neu* gene was able to transform mammary tissue in a single step. On the other hand, studies by, Bouchard et al. [68] demonstrated that expression of c-*neu* was necessary, but not sufficient, to induce the malignant transformation of mammary epithelial cells in mice carrying a MMTV-activated c-*neu* transgene. In the aforementioned experiments, MMTV-directed c-*neu* expression either caused the appearance of synchronous tumors that were polyclonal in origin [67] or the stochastic development of poorly differentiated adenocarcinomas [68]. However, the construct of Muller et al. [67] carried 0.6 kb of additional sequences from the viral Harvey-ras gene between the promoter and the c-*neu* gene, which may explain the alternative phenotype, assuming the construct gave rise to *neu* mRNA with improved translation efficiency or stability. In either case, however, tumor formation was observed in the mammary glands of the mice carrying the MMTV/c-*neu* transgenes. Expression of the β-casein-directed *neuT* transgenes, on the other hand, was insufficient to allow for tumor formation. To determine whether the construct was functional, it was introduced into HC11 cells. Approximately $10^7$ HC11 cells carrying the transfected *neuT* construct were injected subcutaneously into six nu/nu mice. After approximately 3 months, 4 of 6 animals displayed palpable tumors, the largest measuring 27 × 21 × 45 mm in size. Preliminary analysis of tumor biopsies has not demonstrated elevated levels of *neu* mRNA (Greenberg and Rosen, unpublished data). It is interesting to note that a β-casein-genomic *int-2* construct was similarly expressed at very low levels in transgenic mice. However, when HC11 cells transfected with the *int-2* transgene were injected into six nu/nu mice, three of the mice developed tumors within 3 months, the largest measuring 20 × 18 × 11 mm. At 1 year of age, tumor formation has not yet been observed in male or female mice carrying the *int-2* transgene.

From the discussion presented in this section, it appears that −524/+490 β-casein-oncogene fusion constructs, although functionally expressed when transfected into HC11 cells, were unable to facilitate transformation of the mammary gland in transgenic mice. These findings contrast with those obtained with similar constructs using the MMTV-LTR, probably because the MMTV-LTR expresses earlier in development and in less differentiated cell types than the β-casein promoter. This illustrates the importance of using regulatory elements exhibiting different temporal and spatial patterns of mammary-specific expression in transgenic studies. One goal, in particular, will be to isolate and characterize promoter sequences that function specifically in the endbuds of the developing mammary gland, since this is the site of accelerated, rapid growth and proliferation preceding lactation.

In summary, we have presented our current understanding of casein gene expression at the molecular level, and in doing so we have tried to

emphasize both the strengths and weaknesses of the technological and biological systems available for these studies. We have no doubt that the next decade will bring many new and exciting discoveries in the field of mammary-specific gene expression.

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# 19. The whey acidic protein

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## 1. Introduction

Although genes encoding caseins and whey proteins have some control mechanisms in common, namely, their mammary specificity, other aspects of their regulation are quite different. In particular, induction of gene expression during pregnancy and the dependence on steroid and peptide hormones for maxium mRNA accumulation differ between the casein and whey protein genes.

With the introduction of transgenic mice it became possible to study milk-protein gene expression not only in artificial tissue culture cell systems, but also in the context of the whole animal during pregnancy and lactation. Here we review in vitro and transgenic experiments that provided the basis for our understanding of the regulation of the whey acidic protein (WAP) gene. Furthermore, this review, in conjunction with Chapter 18, emphasizes different regulatory pathways and control elements used by the casein and WAP gene in mammary cells during pregnancy and lactation.

## 2. The whey acidic protein

The whey acidic protein (WAP), which was first described in rat milk in 1978 [1], is an abundant milk protein in mice [2–4], rats [1,3,5–7], rabbits [8,9], and camels [10], but has so far not been identified in milk from other species. WAP is characterized by having a conserved cysteine pattern that identifies it as a member of the 'four-disulfide core' protein family [4]. The amino acid conservation between the WAPs from mouse, rat, rabbit, and camel is shown in Figure 1.

Aside from serving as a nutrient source for the suckling young, there has been no physiological role assigned to WAP. It does, however, share amino acid sequence homology with the family of mucous protease inhibitors (MPI) [11] shown in Figure 2. It would not be surprising if WAP had a function besides being a nutrient. Alpha-lactalbumin, for example, was also

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 


*Figure 1.* Sequence comparison of the WAP from mouse [4], rat [6], rabbit [9], and camel [10]. Amino acids identical between all four species are shaded. The arrows refer to the intron-exon boundaries of the mouse and rat WAP genes [12].

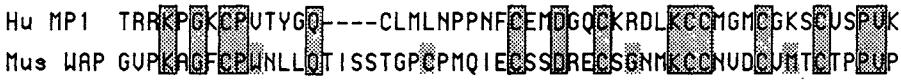


*Figure 2.* Sequence comparison between the third exon of the mouse WAP [12] and human MPI [11] genes. The shaded amino acids in the mouse WAP indicate those that are identical between all WAPs (see Figure 1).

thought to only serve as an amino acid nutrient source before its role as a cofactor of galactosyltransferase was discovered.

## 3. The whey acidic protein gene

### *3.1. Structure*

Genomic DNA encoding mouse [12], rat [12], and rabbit [13] WAP has been isolated and characterized, and several aspects of their expression have been investigated. The WAP gene, in all three species, contains four exons, with exons 2 and 3 encoding the two cysteine domains [12,13]. The sizes of exon 2 and 3, as well as the positions of intron/exon junctions, suggest that they arose through duplication of a primordial exon. Cysteine domain 2 shows a high degree of homology, not only between WAPs from mouse, rat, rabbit, and camel (Figure 1), but also striking similarities to the elastase inhibitor domain in the human mucus protease inhibitor (MPI) ([11] Figure 2). The cysteine residues are identical among the four WAPs and are also highly conserved in human MPI. This, together with the conserved structure

of exon 3 of the WAP and MPI genes, suggests a close evolutionary, and possibly functional, relationship between the two genes.

Since no WAP, or a protein equivalent to it, has been found in milk from humans or pigs, we tested whether the porcine homologue to human MPI, namely, porcine antileukoproteinase (pALP) [14], was expressed in mammary tissue. Although pALP was expressed at high levels in uterine tissue from pregnant animals [14], no transcripts were detected in mammary tissue from virgin, pregnant, or lactating pigs (our unpublished observation). Thus, two members of a gene family that have maintained a high degree of conservation in the protein coding region have acquired different tissue specificities.

The promoter/upstream region of the mouse WAP gene has been analyzed for sequences that interact with nuclear proteins isolated from mammary epithelial cells and elements that activate transcription in vitro and in cell lines. Nuclear proteins isolated from mammary tissue of virgin, midpregnant, and lactating mice recognize control sequences in the mouse WAP gene promoter, including NF1 consensus sequences and an unusual TTTAAA box [15]. Nuclear proteins from nonmammary gland cells (i.e., liver, kidney, etc.) also bind to those sequences in the WAP gene promoter. Binding of those ubiquitous nuclear proteins is clearly insufficient in itself to confer tissue specificity to the WAP gene. Therefore, it is possible that additional, as yet unidentified, binding sites are responsible for dictating tissue specificity. Alternatively, ubiquitous transcription factors may only have access to WAP gene sequences in mammary epithelial cell nuclei. The latter hypothesis is supported by two lines of evidence. First, the WAP gene promoter is efficiently transcribed in vitro by nuclear proteins from nonmammary cells [16], and second, sequences upstream of the TTTAAA box can activate heterologous promoters upon transfection into non-mammary tissue culture cells [16].

### *3.2. Regulation*

Expression of the WAP gene is thought to be specific to mammary epithelial cells. Tissue specificity has been established in lactating but not nonlactating animals [17]. The steady-state level of mouse WAP mRNA increases several thousandfold between the virgin state and midlactation [17–20]. Induction during pregnancy and lactation depends not only on the presence of various hormones, including hydrocortisone and prolactin [18–20], but also on some undefined feature(s) of cell-cell interactions between the mammary epithelium and surrounding stromal cells [21]. Whereas mammary epithelial cells grown on floating collagen gels or a basement membrane matrix will secrete caseins [22,23], the WAP gene is completely silent [22]. Recently Chen and Bissell [24] succeeded in culturing primary mouse epithelial cells on basement membrane matrices, which resulted in the formation of three-dimensional alveoli-like structures. Under such conditions the WAP gene

was expressed, emphasizing the importance of cell-cell communications in the mammary gland.

In comparing WAP to other major milk-protein genes, its expression pattern during pregnancy differs from that of caseins. Steady levels of WAP RNA increase sharply between days 15 and 16 of pregnancy in mice [18,19,25], a period during which the levels of placental lactogens are near maximum but prolactin levels are low [26]. In contrast, β-casein gene expression is induced at day 10 of pregnancy [25], coincident with the increase in placental lactogens. In spite of their different temporal patterns of expression during pregnancy, expression of both the WAP and β-casein genes in mammary explants from mice at midgestation required insulin, hydrocortisone, and prolactin [19,25,28]. Taken together this suggests that expression of various milk-protein genes is subject to different control mechanisms.

Whether WAP gene induction during pregnancy and upon hormonal stimulation in organ explant cultures is regulated on a transcriptional or post-transcriptional level remains controversial. Nuclear run-on transcription data suggest that trancriptional rates of the WAP gene in primary mammary epithelial cells [24] and a mammary epithelial cell line [27] are not affected by hormone treatment. However, studies in transgenic animals indicate that transcriptional induction is the key to increased steady-state

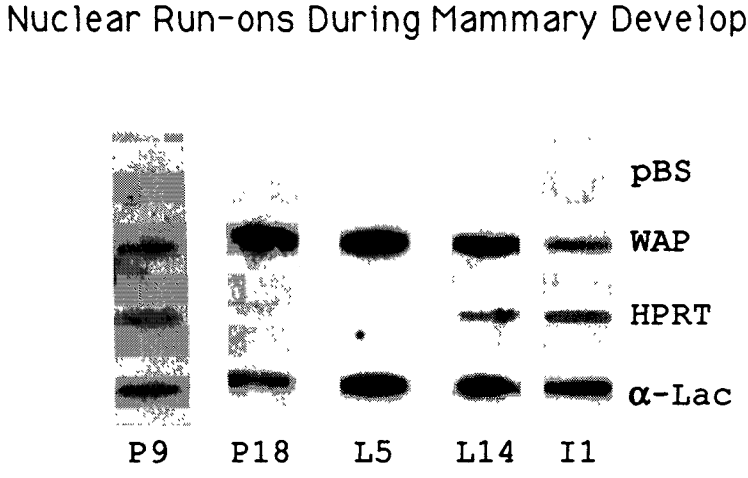


*Figure 3.* Nuclear run-on transcription in mammary tissue from pregnant and lactating mice. Nuclei were prepared from mammary tissue at days 9 (p9) and 18 (p18) of pregnancy, day 5 (L5) and 14 (L14) of lactation, and day 1 (I1) of involution. Nascent RNA molecules were labeled, and RNA isolated from these nuclei was hybridized to plasmids immobilized on a nylon membrane. pBS = plasmid without insert; α-lac = plasmid with an α-lactalbumin cDNA; HPRT = plasmid with an HPRT cDNA insert; WAP = plasmid with a mouse WAP cDNA insert.

levels of WAP mRNA (see later). To determine whether increased steady-state levels of WAP mRNA can be correlated with increased transcription rates, we performed nuclear run-on experiments with mammary tissue from different stages of pregnancy and lactation (Figure 3). About a fivefold increase in transcription rate was observed between day 9 of pregnancy and day 14 of lactation. During this period a several thousandfold increase of WAP mRNA is observed, suggesting that WAP gene induction is primarily due to post-transcriptional events. However, an alternative interpretation of these results could be that the conditions under which run-on assays are performed do not allow for an accurate measurement of transcription rate. Possibly transcription complexes encompassing the WAP gene are not sufficiently stable to be maintained in nuclei from isolated cells. Disrupting cell-cell interactions of mammary tissue is known to abrogate WAP gene expression. If expression of milk-protein genes is dependent, in part, on associations of their regulatory sequences with the nuclear matrix, and the nuclear matrix architecture is dependent on extracellular matrix, disrupting cell-cell interaction could have a profound and immediate effect on milk-protein gene transcription initiation and elongation. Therefore, transgenic animals appear to be, at least at the present time, the optimal system to study WAP gene regulation.

## 4. Transgenic animals

### *4.1. Expression of WAP transgenes in mice and pigs*

Since no adequate tissue culture cell line is available, transgenic animals have been used to study WAP gene regulation during pregnancy and lactation and during hormonal induction in vitro in mammary explant cultures. Regulation of a mouse WAP transgene, which contained 2.6 kb of 5′- and 1.6 kb of 3′-flanking sequences, was studied in transgenic mice and pigs [28–30]. Of the 13 lines of transgenic mice examined, six expressed the transgene in mammary tissue during lactation at levels between 3% and 54% of the endogenous gene [28]. In contrast, all animals from six lines of transgenic pigs expressed the mouse WAP in their milk at levels similar to the endogenous gene in mice [29,30]. Since pigs do not have an identifiable WAP gene but efficiently express a mouse WAP transgene, it is clear that the molecular basis of mammary-specific gene expression is conserved between the two species and between different milk protein genes.

It was surprising, however, to find higher expression across species boundaries (Figure 4). Although the molecular basis of this species difference is unknown, we speculate that transcription elements in the WAP promoter may be more accessible in the pig genome or that possible negative host regulators are missing. Or it could simply be that crossing

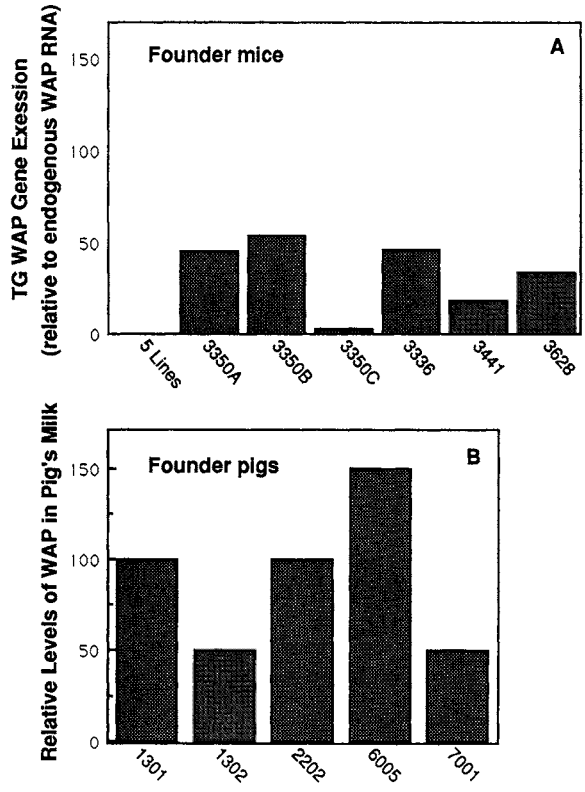


*Figure 4.* Relative expression of mouse WAP transgenes in transgenic mice [28] (A) and pigs (B) [29]. The steady-state level of mouse WAP mRNA in mammary tissue at the peak of lactation (day 10) was arbitrarily set at 100% (A). Similarly, the amount of WAP in mouse milk at the peak of lactation was set at 100% (B). The numbers refer to the individual lines tested.

species barriers minimizes the negative effects of flanking chromatin at random integration sites. In support of this, the sheep β-lactoglobulin gene, which does not have a counterpart in mice, is expressed at high levels in all lines of transgenic mice tested [31].

When the rat WAP gene with only a 950-bp 5′-flanking sequence, was introduced into transgenic mice [32], its expression was similar to that of the mouse WAP transgene. However, the proportion of mouse lines expressing rat WAP was comparable to the proportion of expressing transgenic animals with heterologous regulatory elements. It is possible that the higher percentage of expressing transgenic lines of rat WAP in mice was due to a loss of a repressor in the upstream region. Alternatively, because the rat transgene promoter/enhancer elements were relatively short, head-to-tail arranged transgenes would be more closely spaced, which could result in synergistic activation of the transgenes.

*4.2. Regulation of WAP transgene expression*

Although mammary specificity of WAP transgene expression in transgenic mice was accurately maintained, induction during pregnancy and lactation, and upon hormonal stimulation in vitro, did not follow the endogenous WAP gene [28]. Induction of WAP transgenes during pregnancy preceded that of the endogenous gene in all lines of mice expressing either the mouse [28] or rat WAP transgenes [32]. This suggests that the transgenes may lack repressor elements that normally preclude WAP expression during early stages of pregnancy. Alternatively, the structure of transgene array or surrounding chromatin may interfere with repressor function. Nuclear proteins, isolated from mammary tissue of virgin and early pregnant animals, bound the WAP gene promoter [15], thus supporting the notion that in the absence of a functional repressor, WAP gene expression may be constitutive.

In an experiment where the coding regions of the transgene and the endogenous WAP gene were identical, induction of the two genes differed [28]. The level of endogenous WAP mRNA increased more than 100-fold between day 13 of pregnancy and parturition, but expression of WAP transgenes increased less than tenfold [28] during that same period of time. This clearly suggests that the primary level of regulation is transcriptional and argues against post-transcriptional regulation, as indicated by nuclear run-on assays.

The endogenous WAP gene is expressed at high levels throughout, lactation. However, this was not the case for the expression of the mouse WAP transgene in all lines [28]. In three lines of mice in which the transgene was induced during pregnancy, expression decreased as lactation progressed [28]. A similar decrease in expression was observed in some transgenic mice carrying WAP-GH genes [33]. Since the terminally differentiated mammary epithelial cells die during involution of the gland, one might assume that, except for milk-protein genes, the general level of transcription of cells declines. Furthermore, since the site of transgene integration is thought to be random, it is unlikely that the WAP transgene integrates near other milk-protein genes. Therefore, it is possible that downregulation of the surrounding chromatin could depress expression of the transgene. To determine if 'housekeeping genes' are downregulated, the level of mRNA of six genes was monitored during pregnancy and lactation (Figure 5). Except for the expression of malic enzyme encoding mRNA, which remained constant throughout mammary gland development, all genes showed a slight decrease in expression during lactation. However, the decrease in expression, typically less than 50%, would be insufficient to account for the downregulation of the WAP transgene, especially when compared with the continued increase in endogenous WAP gene expression during lactation. Interestingly, with the onset of lactation, novel RNA species related to keratin Endo B and HPRT were detected (Figure 5). Although the nature of the putative proteins encoded by these novel RNA species is unknown,

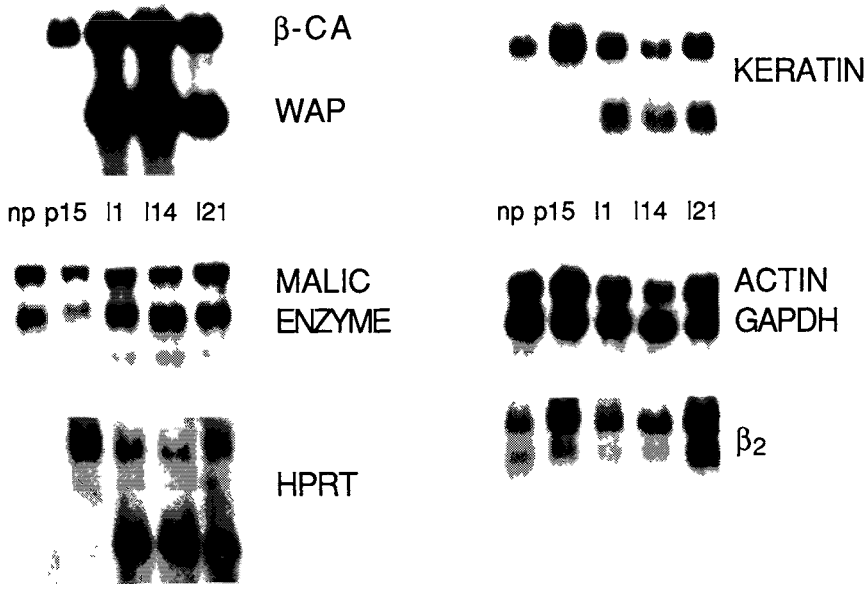


*Figure 5.* Expression of β-casein, WAP, and 'housekeeping genes' in mouse mammary tissue during mammary development. Total RNA isolated from mammary tissue from nonpregnant (np); 15-day pregnant (p15); and 1-day (l1), 14-day (l14), and 21-day lactating (l21) mice were separated in a formaldehyde gel and probed with cDNAs encoding mouse β-casein, WAP, rat actin, mouse keratin endo B [55], rat malic enzyme [56], hamster hypoxantine-phosphoribosyl transferase (HPRT), mouse GAPDH, and mouse $\beta_2$ microglobulin. The malic enzyme and $\beta_2$ microglobulin probes hybridize with two mRNA species each. The sizes of the upper bands in the HPRT and keratin panels correspond to the published sizes. The lower bands are crosshybridizing species that first appear with the beginning of lactation.

they are indicative of the changed physiological state of mammary tissue after parturition. Analysis of endogenous and transgenic WAP expression during pregnancy and lactation provided evidence that the mechanisms of induction differed between the two physiological stages [28]. This is supported by the recent identification of a mammary-specific factor implicated in the regulation of β-casein gene expression that is induced prior to parturition (Bernd Groner, personal communication). While WAP gene expression can be induced by insulin, hydrocortisone, and prolactin in mammary tissue explants from pregnant mice, these hormones were not sufficient to induce or even maintain transgenic WAP mRNA levels in lactating tissue. A similar loss of sensitivity of WAP gene expression to steroid hormones during late pregnancy and lactation was observed by Quirk and coworkers [5]. Therefore some of the elements necesssary for

continued WAP expression during lactation may be located outside the cloned region.

### *4.3. Expression of transgenes containing the WAP promoter*

Mouse WAP gene promoter/upstream sequences have been successfully employed to direct the expression of several genes to mammary epithelial cells [17,18,33–40]. Combined with the data on rat WAP transgenes [32], it can be predicted that the mammary-specific element in the WAP gene promoter must be located within 950 bp of the promoter sequence. Expression levels of hybrid genes containing cDNA or genomic sequences were in general at least an order of magnitude lower than those obtained with a WAP transgene containing the promoter, the transcribed region, and 3′ flanking sequences. Since frequency of expression and transcription efficiency were similar with hybrid genes containing cDNA or genomic sequences, it is likely that the higher activity obtained with genomic WAP transgenes was not due to splicing of the primary transcript per se, but to the presence of transcription elements downstream of the WAP gene promoter, possibly within introns.

Similar to the situation with the WAP genomic transgene, premature induction of hybrid genes was observed during pregnancy [18,33], and in some cases expression decreased during lactation [33]. Hormonal induction of transgenes appeared to be highly dependent on the integration site. While expression of a WAP-tPA transgene did not require the presence of prolactin [18], the genomic WAP transgene [28], and a WAP-*myc* hybrid gene [41] required insulin, hydrocortisone, and prolactin for full induction in mammary tissue from pregnant animals.

### *4.4. WAP gene expression and mammary development*

As previously indicated, WAP is believed to function as a nutrient source for the suckling young. However, it may have other functions, including a role in mammary development. WAP has striking homologies with protease inhibitors from the MPI family [11]. Such proteases and protease inhibitors participate in tissue formation and remodeling [42,43], and it is intriquing to speculate that WAP may be involved in mammary gland development and differentiation. Synthesis of WAP at an inappropriate time during or prior to pregnancy, when it cannot be removed from the site of synthesis, may therefore have adverse effects on development. We have seen evidence of this in our transgenic pig studies [29,30]. Animals from three of six lines of transgenic pigs expressing the WAP gene did not produce sufficient quantities of milk to support normal development of otherwise healthy offspring [29,30]. A histological evaluation of mammary tissue from these agalactic sows revealed abnormalities in mammary gland development [44]; (Figure 6). Constitutive expression of WAP transgenes occurred

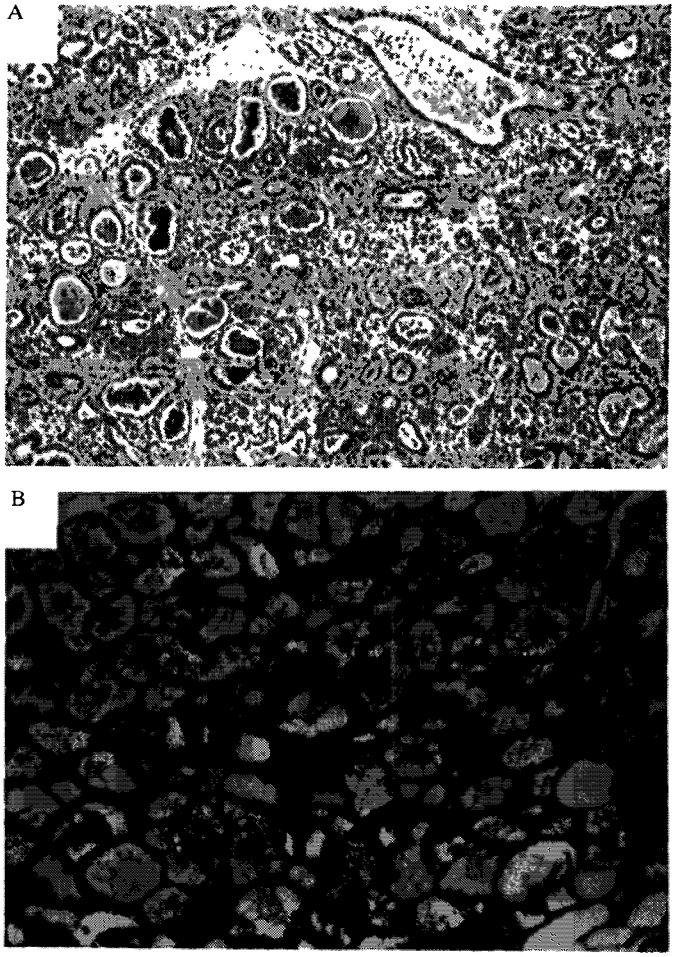


*Figure 6.* Histology of mammary tissue from a postpartum transgenic pig with agalactia (A) and from a nontransgenic pig with no lactation problems [B].

in mammary tissue of sexually immature pigs from lines with agalactia [44], suggesting that precocious WAP expression may have impaired cell proliferation and formation of lobulo-functional alveolar structures during pregnancy.

Agalactia has not been described in transgenic mice expressing high levels of nonmouse milk proteins [31,45–47], or human pharmacologically active proteins [18,33,34,38,48,49]. A phenotype similar to the agalactia described here has been reported in transgenic mice that express the *myc* and *ras* oncogenes [35], the *int-1* growth factor [50], and the *Int-2* gene [51] in their mammary glands. Just as with the transgenic pigs, endogenous milk-protein gene expression was deregulated in transgenic mice expressing the *myc* and

*ras* oncogenes [35]. Phenotypes induced by transgene expression probably reflect properties of the respective organ or cell system. Development of mammary tissue is subject to growth factors and inhibitors. Recent studies indicated that TGF-β1 [52], EGF [53], and mammary derived growth inhibitor (MDGI) [54] are probably natural growth inhibitors in the mammary gland. It is tantalizing to envision that WAP serves a dual role, as nutrient and as regulatory protein.

## Acknowledgment

We thank William Jakoby for creating an environment that allowed us to perform this work.

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# 20. Stromal-epithelial interactions in breast cancer

Kevin J. Cullen and Marc E. Lippman

## 1. Introduction

Breast malignancies are almost exclusively epithelial cancers. Epithelial cells that have undergone malignant transformation arise from ductal or lobular elements within the breast parenchyma. However, no breast cancer can grow beyond the microscopic level simply as a proliferation of malignant epithelium. Clinically significant breast tumors are a complex and heterogeneous mix of epithelium, stromal cells, matrix proteins, and vascular elements. The growth and dissemination of a breast cancer requires complex but poorly understood interactions between these various tumor elements. To grow beyond the microscopic level, a tumor must acquire a blood supply. To acquire invasive or metastatic potential, a tumor must be able to manipulate and degrade surrounding basement membrane and intercellular matrix to escape into lymphatic or vascular channels. These same properties may be necessary for a metastatic tumor focus to develop at a distance from the primary tumor.

While laboratory models of breast cancer have traditionally focused on studies of malignant epithelium, an increasing body of data over the last several years has begun to examine the role of interactions between tumor epithelium and stroma in the genesis and proliferation of breast cancer. While this work is relatively new, it has already added new insights into the control of tumor growth and progression in breast and other malignancies, and may provide new approaches to cancer treatment in the future.

In the first part of this chapter, we will focus on some of the recent studies in the literature that relate to stromal-epithelial interactions in breast cancer. We will conclude this discussion by describing some of our own laboratory studies that relate to this area.

## 2. Stromal/epithelial interactions in breast development

Stromal/epithelial interactions are important in all stages of development for both normal and malignant breast epithelium. Organogenesis in the

*R.B. Dickson and M.E. Lippman (eds.), Genes, Oncogenes, and Hormones: Advances in Cellular and Molecular Biology of Breast Cancer.* 

embryo, pubertal growth in the adolescent, and lactation in the adult are all modulated to a significant degree by these interactions.

Interactions between embryonic mesenchyme and epithelium are an essential component of development and differentiation of the mammary gland [for review, see 1] Numerous studies, primarily in rodent systems, have demonstrated that specific interactions between embryonic mammary epithelium and mammary mesenchyme will induce ductal differentiation. Sakakura and coworkers demonstrated that rudimentary fetal mammary epithelium transplanted into the mammary fat pads of juvenile or adult mice will undergo glandular differentiation. The induction of glandular differentiation is specific for mammary epithelium in this model, since fetal epithelium from lung, pancreas, or salivary gland will not undergo differentiation when transplanted into the mammary fat pad [2].

The same laboratory showed essentially the converse phenomenon by transplanting embryonic mesenchyme from the mammary fat pad or salivary gland into the mammary gland of virgin adult mice. Transplant of embryonic mesenchyme resulted in proliferation and differentiation of glandular epithelium, both in intact and ovariectomized mice. Embryonic mesenchyme from lung, pancreas, or kidney did not have this effect on mammary epithlium [3]. This indicates that there are important interactions between embryonic mammary stromal and epithelial cells that are organ specific and not a generalizable interaction typical of all fetal tissues.

Stromal/epithelial interactions are also important in the mediation of hormonal effects on mammary gland development. In studies on mice, Durenberger et al. demonstrated that during organogenesis androgen treatment caused condensation of mesenchymal cells around the mammary gland bud and is accompanied by death of the glandular epithelial cells. However, mesenchymal cells are necessary for this reaction, because androgens will not cause this effect directly on epithelium in the absence of surrounding mesenchyme. Further, there is no androgen effect on breast epithelium if the epithelial cells are grown with mesenchymal cells from androgen-insensitive mice bearing a testicular feminization mutation. Experimental combination of breast mesenchyme with epithelium from other organs show the mesenchyme-mediated inhibition of epithelial growth is specific for breast epithelium and is not seen in epithelial cells from other organs [4].

Posnatally, stromal/epithelial interactions appear to be important in the steroid-mediated processes of mammary gland maturation through the pubertal period. Using steriod autoradiography in a murine model system, Haslam demonstrated that mammary stromal cells acquire estrogen receptor significantly earlier than mammary epithelial cells, and that stromal cells and epithelial cells both show a mitogenic response to estrogen, prior to puberty at approximately 3 weeks of age [5]. In an earlier study, Narbaitz demonstrated the presence of estrogen receptors in mammary stroma as early as 16 days of fetal development [6]. The functional significance of the stromal response to estrogens in these systems is not clearly defined, however.

Coculture models suggest stromal/epithelial interactions may also have an important function in the regulation of lactation. Reichmann and coworkers isolated a mouse mammary cell line, designated IM-2, which contained a mixture of fibroblast and epithelial elements. IM2 synthesized significant quantities of the milk protein β-casein in response to stimulation with prolactin and insulin. However, when the fibroblasts and epithelial cells were isolated and grown separately, neither was able to produce β-casein in response to the same lactogenic stimulus. When the two sublines were cocultured, the epithelial cells were again able to produce milk protein in response to prolactin and insulin stimulation. The authors also noted that when the epithelial cells were grown alone in culture, they formed simple monolayers. However, when cocultured with breast fibroblasts, the epithelial cells formed three-dimensional duct-like structures in close approximation to the breast fibroblasts. Laminin was found at the interface of the fibroblast and epithelial cells, and was closely correlated with the ability of the epithelial cells to produce β-casein. The authors hypothesize that this basal lamina is necessary to produce differentiation and casein secretion in the epithelial cells [7].

## 3. Stromal matrix proteins and proteases

Extracellular matrix produced by breast stromal cells is not simply a passive scaffolding for epithelial cells. Matrix proteins provide both a mechanical boundary and support for breast epithelial cells. They also appear to influence the orientation and differentiation of normal breast epithelium. Wicha et al. demonstrated that rat mammary epithelial cells grown on purified acellular matrix derived from host mammary glands showed significant differentiation, evidenced by a marked increase in the production of α-lactalbumin and microvilli formation. These effects were not seen when cells were grown on simple collagen gels [8].

Li et al. performed similar studies in which mouse mammary epithelial cells grown on a reconstituted basement membrane preparation (matrigel) showed a marked increase in the levels of β-casein production as well as formation of structures resembling ducts, lumina, and alveoli. Ultrastructurally, the epithelial cells resume a polar orientation (lost during growth on plastic) with apical secretion of milk proteins. These effects were not seen with cells grown on type IV collagen or fibronectin alone [9]. Similarly, the isolated matrix proteins heparan sulphate proteoglycan and laminin induced small increases in milk protein mRNA but did not induce the full functional and histological differentiation seen when cells were grown on matrigel [10].

While containing abundant basement membrane, matrigel is a complex material that has not been completely characterized and contains numerous proteins that may impact on epithelial cell behavior. Nevertheless, the

results of both these studies demonstrate that important interactions between epithelial cells and stromal matrix can have a profound impact on epithelial cell gene expression and overall cellular function.

More recently the same group has mammary epithelial cells cultured on floating type I collagen gels that are able to synthesize and deposit new basement membrane, while the same cells cultured on plastic are not. However, mRNA levels of basement membrane components (laminin, fibronectin, and type IV collagen) were highest in cells grown on plastic. The authors hypothesize that mRNA expression of component proteins is not sufficient for the production of functional basement membrane and that a specific environment that permits polar differentiation of epithelium is necessary for membrane formation [11]. Stromal regulation of the functional differentiation of mammary epithelium, therefore, appears to be the result of induction of specific gene expression in the setting of a specific physical and chemical microenvironment.

Other matrix proteins may also have a significant impact on epithelial cell structure and function. Tenascin is a glycoprotein that interferes with cell adhesion and may participate in the process by which tumor cells are released from their host environment and metastasize to other sites [12]. Chiquet-Ehrismann et al. demonstrated that conditioned media from MCF-7 cells was able to induce tenascin secretion by chick embryo fibroblasts. An antibody to TGF-β1 was able to block the induction. The authors hypothesize that TGF-β1, a product of breast tumor epithelial cells [13], induces stromal production of tenascin, which in turn can promote epithelial cell dissociation and metastasis [14]. Scatter factor, also known as hepatocyte growth factor, is a basic protein produced by fibroblasts that induces the separation of contiguous epithelial cells and increases the motility of epithelial cells in culture [15]. While the scatter factor has been hypothesized to be important in embryogenesis, its role in tumor development and progression is not clear.

In order for a tumor to grow and metastasize, it must be able to degrade the extracellular matrix of adjacent normal tissue in order to escape the confines of its local environment. A number of proteolytic enzymes produced in tumors have been identified that may contribute to this process. Among these are urokinase-type and tissue-type plasminogen activator [for review, see 16], type IV collagenase, and others. Type IV collagenase is part of a family of enzymes known as metalloproteinases, which are balanced in vivo by the specific tissue produced inhibitors, TIMP-1 and TIMP-2 [for review see 17].

Plasminogen activator has been described as a component of many human tumors. The enzyme converts plasminogen to the active protease plasmin, which has significant proteolytic and thrombolytic activity. Some systems have suggested that tumorigenicity is enhanced by plasminogen activator production [18]. MCF-7 cells increase the production of plasminogen activator more than two times over control levels following treatment with

physiologic concentrations of estradiol [19]. Significantly higher concentrations of the androgen testosterone ($10^{-6}$ M) showed a similar effect, while antiestrogens were able to inhibit plasminogen activator production by MCF-7 cells [20].

Analysis of human breast tumor explants showed that plasminogen activator activity was significantly increased in approximately 50% of tumors that were both estrogen and progesterone receptor positive. No estradiol-mediated increase in plasminogen activator activity was seen in any of the tumors that were not positive for both estrogen and progesterone receptors [21]. Of the two principal types of plasminogen activator, the tissue type and the urokinase type, only the tissue-type plasminogen activator appears to be related to estrogen receptor and estradiol sensitivity [22].

More recently, Duffy and coworkers analyzed the expression of urokinase-plasminogen activator as a prognostic indicator in breast cancer. In this study, high levels of urokinase plasminogen activator (UK-PA) were associated with early relapse and shorter overall survival. Multivariate analysis showed that UK-PA expression was an independent risk factor for relapse when compared with tumor size and axillary nodal status [23].

Collagenase activity has been examined in a number of cell culture and tumor biopsy experiments. MCF-7 cells secrete collagenases, which are able to lyse Type I and Type IV collagen. Type I collagen is present in tissue stroma and in bone, while Type IV is the principal form seen in cellular basement membranes. Estradiol treatment resulted in a two- to threefold increase in collagenase activity in MCF-7 cells [24].

Immunohistochemical analysis of breast tumor specimens with an antibody against a type IV collagenase showed intense epithelial staining in 25 of 25 cases of invasive carcinoma studied. Interestingly, no fibroadenomas, fibrocystic disease biopsies, or normal breast specimens were positive in this study [25]. Another study of type I collagenase activity in breast tumor specimens showed uniformly high secretion by fibroadenomas compared with benign fibrocystic lesions, while carcinoma specimens showed a wide range of collagenolytic activity, which did not correlate with the histologic type [26].

Monteagudo and coworkers examined in situ expression of type IV collagenase in normal and maligant breast. Strong immunostaining was seen in the myoepithelial cells of normal breast, but less frequently in luminal epithelial cells. However, epithelial cells in intraductal and invasive cancers, as well as metastatic lesions, were positive in the majority of cases, as were metastatic lesions [27]. Similarly, expression of Type IV collagenase and laminin receptor appears to correlate with malignant progression in breast cancer and other tumors. Expression of both proteins was significantly higher in invasive cancers than in normal epithelium or in situ cancers [28]. Similar results were seen in gastric and colon carcinomas. A recent retrospective study of type IV collagenase expression in node-negative breast cancer by Di Fronzo et al. did not demonstrate a correlation between

expression of type IV collagenase and relapse-free or overall survival in these patients, although collagenase expression was an important indicator of local-regional progression of disease in this study [29].

Recently, Basset et al. have identified and cloned a novel metalloproteinase that is produced by stromal cells at the advancing margin of invasive breast carcinomas. Using a subtractive cloning technique to a cDNA library derived from a breast tumor surgical specimen, rather than a cell line, they identified several genes that were expressed in tumor but not in a fibroadenoma. The gene sequence identified predicted a 488-residue protein, stromelysin 3 (ST3), that showed significant homology to several metalloproteinases. In situ hybridization studies showed that message was not seen in breast tumor epithelial cells, but was seen as high levels in stromal cells immediately surrounding tumor epithelial islands. No signal was detected in stroma surrounding in situ carcinomas. Cellular growth factors known to be secreted by breast tumor epithelial cells, including PDGF and basic FGF, caused a significant increase in ST3 expression by human fetal fibroblasts [30].

These studies demonstrate in both clinical tumors and cell culture that the ability to digest basement membrane collagen and other collagen substrates is important in breast tumor invasion and metastasis. The tumor matrix environment is plastic and can be modified by stromal and epithelial cell products in ways that promote tumor growth and progression.

## 4. Stromal cell phenotypic variation

The thesis of this discussion is that stromal/epithelial cell interactions have great significance, both in the regulation of normal mammary gland development and in the regulation of breast cancers. A fundamental question that remains only partially answered is whether stromal cells within a breast malignancy are phenotypically and genotypically different from stromal cells in the normal breast tissue, or elsewhere in the organism. Breast cancers are almost exclusively epithelial rather than mesenchymal tumors. The stromal cells within a tumor are not in and of themselves malignant, but several groups have produced data that suggest the fibroblasts grown from a breast cancer may be fundamentally different from the fibroblasts elsewhere in the breast. Indeed, some groups have suggested that those diferences are more generalized and that skin fibroblasts from patients with breast cancer or other tumors may be different from skin fibroblasts of controls without cancer.

A subset of fibroblasts within breast lesions demonstrate elements of smooth muscle differentiaion [31] and have been designated myofibroblasts. They are distinguished from myoepithelial cells by the presence of vimentin expression and the absence of keratin. Likewise they are distinguished from smooth muscle cells immunohistochemically by the absence of desmin [32]. Using immunostains for α-smooth muscle actin, Sappino and coworkers

showed actin expression in the stromal tissue of 11 of 11 breast cancers, but none of seven normals. Intermediate levels of stromal actin expression were seen in fibroadenomas and other benign lesions [33]. The mechanisms responsible for the genesis of this smooth muscle differentiation are not clear, nor is the biologic significance of that phenotype. However, the data do suggest that stromal cell populations within breast tumors may be functionally distinct from normal breast stroma.

Numerous groups have demonstrated phenotypic differences between fibroblasts from tumors or skin of cancer patients compared with normals. Pfeffer et al. noted abnormal growth behavior in skin fibroblasts derived from patients with familial polyposis of the colon, a condition associated with a very high predisposition for tumor formation [34]. Azzarone et al. have demonstrated abnormal growth behavior in skin fibroblasts derived from breast cancer patients compared with normal controls. Thymidine labeling studies showed higher levels of thymidine uptake in breast tumor fibroblasts that had reached confluence when compared with controls [35, 36]. Schor et al. have shown fetal patterns of motility in collagen gels for skin fibroblasts derived from patients with a number of malignancies, including breast cancer, melanoma, and Wilms' tumor [37]. Briefly, they observed that normal skin fibroblasts will migrate into collagen gels when plated at low density, but that this migration is inhibited when the cells are plated at high density. Fetal fibroblasts, transformed fibroblasts, and skin fibroblasts from patients with various cancers will migrate into collagen gels, even when plated at high density. By quantifying the difference in collagen migration between fibroblasts plated at low and high density, a single value, designated the cell density migration index (CDMI), is measured. They hyothesized that the persistence of this fetal fibroblast motility pattern is associated with an increased risk of subsequent tumor development [38] and more recently have isolated a 50 to 60-kDa protein produced by fetal fibroblasts as well as skin fibroblasts from cancer patients, but not normal adult cells, that stimulates fibroblast miration fibroblasts [39,40]. More recently, they have reported this protein was present in the serum of 83% of breast cancer patients evaluated prior to therapy, and in 93% of patients who had received treatment for their cancer and were felt to be free of disease. In comparison, only 10% of normal controls without cancer had detectable migration-stimulating activity in their serum [41].

These studies raise the interesting possibility that systemic phenotypic differences exist between stromal fibroblasts in cancer patients and in normals. Whether these differences may contribute to the genesis or promotion of breast cancer or other tumors is not known. Additionally, it remains to be seen whether these differences can be used to identify patients who may be at increased risk for breast cancer.

## 5. Stromal-mediated mitogenesis of normal and malignant breast epithelium

Data from several in vivo and in vitro model systems suggest that stromal influences enhance both tumor formation and growth rate in a number of epithelial cancers including breast. Adams et al. reported that conditioned medium from fibroblasts derived from benign and malignant breast tumors had a marked stimulatory effect on MCF-7 cell in culture. Interestingly, the opposite effect was seen with conditioned medium from fibroblasts derived from benign breast tissue, which actually inhibited the growth of MCF-7 cells [42].

Transformed fibroblasts have been shown to accelerate the growth of several tumor cell types in tissue culture and nude mouse models. Camps and coworkers demonstrated that coinoculation of epithelial tumor cell lines with transformed fibroblasts shortened the latency period and increased the growth rate of tumors in athymic animals. Tumors formed in 0 out of 16 nude mice injected with a human prostate cancer line PC-3. However, tumors were seen in 25 out of 28 mice coinoculated with transformed rat prostatic fibroblasts. Similar results were seen when the same fibroblasts were coinoculated with breast or bladder tumors cells. Coinoculation with nontransformed mouse fibroblasts (NIH-3T3) or lethally irradiated fibroblasts also produced similar increases in tumor take, but histologic analysis of the tumors showed that there was a much more prominent stromal component in the tumors inoculated with transformed fibroblasts [43].

Horgan et al. have reported similar findings using human breast and skin fibroblasts coinoculated with MCF-7 cells in nude mice. MCF-7 cells coinjected with breast fibroblasts formed tumors in 94% of animals, compared with 49% of animals injected with MCF-7 cells alone. Tumors from animals inoculated with fibroblasts weighed four times more than tumors from controls after 5 weeks of growth. No difference was seen in tumor take rate or the size of tumors whether the breast fibroblasts were derived from tumor, uninvolved breast tissue from cancer patients, or reduction mammoplasty. Skin fibroblasts coinoculated with MCF-7 cells increased the tumor take rate, but accelerated growth less than breast fibroblasts [44,45].

These data in nude mice suggest that some of the tumor-promoting effects of fibroblasts are not tissue specific and may in part be due to ill-defined host responses to the various cell types inoculated. However, the notion that in tissue culture conditioned medium from normal breast fibroblasts can inhibit breast tumor epithelial growth while conditioned media from tumor fibroblasts is stimulatory suggest that there are a range of distinct phenotypic populations of breast fibroblasts and that soluble factors produced by these cells may have a profound impact on the growth and behavior of malignant breast epithelium.

Several groups have shown that interactions between breast epithelium and surrounding stroma are important in the mitogenic response to

estrogens as well. In mouse cell-culture experiments, estrogen-induced proliferation of mammary epithelial cells is seen only when epithelial cells are cultured in the presence of mammary stromal cells [46]. Furthermore, there appears to be more than one mechanism by which stromal cells participate in the mammary epithelial response to estrogen. Mouse mammary epithelial cells treated with estradiol in the presence of killed fibroblasts or fibroblast-conditioned media show induction of progesterone receptor, but not increased DNA synthesis nor epithelial cell proliferation. Estradiol-induced cell proliferation in epithelial cells requires direct contact with live fibroblasts. Under these conditions, epithelial cells, in turn, promote estrogen-induced stimulation of fibroblast cell proliferation [47], suggesting that the stromal/epithelial interactions involved in mammary growth regulation are bidirectional.

A further mechanism for stromal regulation of the epithelial response to estrogen was proposed by Adams and coworkers, who noted that breast-fibroblast-conditioned media induce 17β-estradiol dehydrogenase activity, increasing conversion of estrone to estradiol [40,48]. Both lines of evidence indicate that stromal/epithelial interactions may alter the bioavailability and physiologic role of estrogen in normal and malignant mammary epithelium.

Finally, stromal/epithelial interactions may have a role in mediating the transformed phenotype associated with certain oncogenes. Valverius and coworkers studied a human mammary epithelial cell (A1N4) infected with retroviral vectors for either c-*myc* or SV40T. Epithelial cells that expressed *myc* or SV40T did not grow in soft agar when plated by themselves. However, when these cells were plated in agar along with cultured diploid mammary fibroblasts, they formed growing colonies. Similarly, conditioned media from breast fibroblasts stimulated epithelial colony formation in soft agar, as did purified EGF, TGF-α, or basic FGF [49]. Parental A1N4 cells did not form colonies under any of these conditions. These data suggest that soluble growth factors, provided by stromal fibroblasts, may be important in establishing and maintaining the transformed phenotype induced by protooncogenes and oncogenes.

## 6. Growth-factor-mediated stromal/epithelial interactions in breast cancer

It has not been fully defined how communication between different cells within a tumor is controlled, but peptide-growth-factor-mediated signals between cell types have been shown to be important in several areas. As cited above, the secretion of PDGF by tumor epithelial cells can be a potent stimulus for the growth of stromal tissues [50], while the production of fibroblast growth factors by tumor epithelium promotes the growth of vascular tissues necessary to provide the tumor with a blood supply [51,52]. Comparatively little is known about how growth factor production by tumor

stromal elements can modulate the growth of transformed tumor epithelial cells.

Our group became interested in the topic of stromal growth factor production through previous work on the expression and function of the insulin-like growth factors, IGF-I and IGF-II, in human breast cancer. The insulin-like growth factors have been extensively characterized and have been shown by several groups to be potent mitogens for breast cancer epithelial cells [53–56]. While the overwhelming majority of surgical breast tumor specimens we examined expressed both IGF-I and IGF-II mRNA, no cultured breast tumor epithelial cells were found to express authentic IGF-I mRNA, using a highly specific RNAse protection assay [57]. Similarly, significant IGF-II mRNA expression was found in only a single malignant breast epithelial cell line, T47-D [54]. In situ hybridization studies demonstrated that IGF-I mRNA could be detected in the stroma of normal tissue at a distance from the breast tumor, but not in the stromal or epithelial cells of the tumor itself [9]. Since we detected both IGF-I and IGF-II mRNA in the majority of tumor surgical specimens but only very rarely in cultured tumor epithelial cells, and since the surgical specimens analyzed include a mix of various tumor and nontumor elements, our results raised the possibility that the IGF message we detected in surgical specimens arose primarily from tumor stroma and not from tumor epithelial cells. This suggested that stromal IGF might be acting primarily as a paracrine stimulant for breast tumor epithelium. The notion that IGF expression might be primarily stromal in origin is supported by the fact that IGF overexpression has been reported in a number of sarcomas [58,59], which are tumors of mesenchymal origin without a predominant epithelial component.

In order to postulate a paracrine stimulatory role for IGF-I or IGF-II in breast cancer, breast cancer epithelial cells must have appropriate receptors for these ligands. Several groups have examined breast cancer cell lines for the presence of IGF receptors by binding competition studies and affinity crosslinking. By these methods, both type I and type II IGF receptors are present on the cell surface of a number of breast cancer cell lines [60]. Similar studies have demonstrated the presence of type I and type II IGF receptors in breast tumor tissues [61,62]. Type I receptors have been demonstrated in benign breast lesions as well [63]. However, ligand binding studies are difficult to interpret in this system, because of receptor cross-reactivity and because of the presence of several IGF binding proteins, some of which may be membrane associated. Also, these studies do not indicate which receptor or receptors in the system are important for mediating the biological responses induced by the IGFs.

In an effort to help clarify these questions, we examined breast cancer cell lines and tumor samples for mRNA expression of the insulin receptor as well as the type I and type II IGF receptors. All breast cancer cell lines examined expressed mRNA for the type I IGF receptor, type II IGF receptor, and the insulin receptor. In addition, 6 of 7 breast tumor biopsy

specimens expressed type I receptor mRNA, and 11 of 11 tumor specimens expressed type II IGF receptor mRNA. Binding studies confirmed previous research, demonstrating high-affinity type I and type II IGF receptors in MCF-7 cells. In the same study, we were able to demonstrate that α1R3, a monoclonal antibody that blocks binding to the type I IGF receptor, blocked the mitogenic effects of both IGF-I and IGF-II but not insulin. αIR3 also was able to block greater than 80% of radiolabeled IGF-I binding to MCF-7 cells, but did not block radiolabeled IGF-II binding. This suggests that although both type I and type II receptors are expressed in breast cancer cells, the mitogenic response to both IGF-I and IGF-II is mediated by the type I receptor [64]. Osborne et al. reported similar findings [65]. Other studies have shown that blockade of the type I IGF receptor with this same antibody can reduce the rate of cell growth of MCF-7 cells grown in serum [66], as well as inhibit the formation of MDA MB-231 tumors grown in nude mice [67]. Since we did not find that this cell line has mRNA for either IGF-I or IGF-II, one possible interpretation of these results is that the αIR3 inhibited the effects of IGF produced by mouse stroma. While all of these studies indicate that the type I receptor is integrally involved in the tumor cell growth stimulated by IGF-I and IGF-II, the function of the type II receptor in this system is not clear.

On the basis of these observations, we performed two sets of experiments. First, we examined the expression of IGF-I and IGF-II, as well as members of several other growth factor families, in primary cultures of stromal fibroblasts derived from benign and malignant breast lesions. On the basis of these experiments, we were able to identify stromal growth factors that might be important in the overall growth regulation of benign and malignant breast lesions. These experiments are described in detail elsewhere [68].

Most of the growth factors examined showed consistent patterns mRNA expression, regardless of whether the fibroblast line studied originated from a benign or malignant lesion. All fibroblasts examined expressed PDGF A chain, while none expressed the PDGF B chain. All expressed TGF-β1, while none expressed TGF-α. All expressed both basic FGF and FGF-5 mRNA. Only the pattern of expression for the insulin-like growth factors showed differences that correlated with whether the fibroblast studied originated from a malignant or benign lesion. IGF-I expression was seen in 7 out of 8 fibroblasts derived from benign lesions, but in only 1 of 9 lines derived from malignant tumors. IGF-II expression was roughly the opposite, seen in only 1 of 9 lines derived from benign lesions, but present in the majority (5 of 9) of lines derived from malignant tumors. For both IGF-I and IGF-II, the differences in mRNA expression between fibroblasts derived from benign and malignant lesions were statistically significant despite the small total number of samples. There did not, however, appear to be any readily identifiable correlation between IGF expression and other prognostic indicators, such as estrogen and progesterone receptor status or nodal

*Table 1.* Growth factor mRNA expression by breast fibroblast cell lines

| Fibroblast | IGF I | IGF II | PDGF A | PDGF B | TGF α | TGF β1 | Basic FGF | FGF 5 | Source |
|---|---|---|---|---|---|---|---|---|---|
| 191 | + | − | + | − | − | + | + | + | Red. mammo. |
| 365 | + | − | + | − | − | + | + | + | Red. mammo. |
| 429 | + | − | ND | ND | ND | ND | ND | ND | Red. mammo. |
| 446 | ND | − | ND | ND | ND | ND | ND | ND | Red. mammo. |
| 879 | + | − | ND | ND | ND | ND | + | + | Fibroadenoma |
| 987 | + | − | ND | ND | ND | ND | + | ND | Fibroadenoma |
| 999 | − | − | + | − | ND | ND | ND | ND | Fibroadenoma |
| 1034 | + | − | + | − | − | + | + | + | Fibroadenoma |
| 1097 | + | + | ND | ND | ND | ND | + | ND | Red. mammo. |
| 197 | ND | ND | ND | ND | ND | ND | + | + | Tumor |
| 406 | + | − | ND | ND | ND | ND | + | + | Tumor |
| 559 | − | + | + | − | − | + | + | + | Tumor |
| 788 | − | + | + | − | − | + | ND | ND | Tumor |
| 906 | − | + | + | − | − | + | + | ND | Tumor |
| 926 | − | − | ND | ND | − | + | + | + | Tumor |
| 971 | − | + | ND | ND | ND | ND | ND | ND | Tumor |
| 974 | − | − | + | − | − | + | + | + | Tumor |
| 977 | − | + | + | − | − | + | ND | ND | Tumor |
| 995 | − | − | + | − | − | + | + | + | Tumor |

IFG-I = insulin-like growth factor I; IGF-II = insulin-like growth factor II; PDGF A = platelet-derived growth factor A chain; PDGF B = platelet-derived growth factor B chain; TGF α-transforming growth factor-alpha; TFG-β1 = transforming growth factor-beta 1; basic FGF = basic fibroblast growth factor; FGF-5 = fibroblast growth factor 5; ND = not done.

involvement. Analysis of one of the IGF-II positive tumor derived fibroblast lines demonstrated secretion of biological significant quantities of IGF-II protein, confirming the RNA data. The RNA data from this study are summarized on Table 1.

Of the growth factors produced by the fibroblasts examined in this study, IGF-I and IGF-II have the most clearly defined mitogenic effects on tumor epithelial growth. TGF-β1 appears to be most active as an inhibitor of breast tumor epithelial cell growth [69,70]. Breast tumor epithelial cells in vitro lack receptors for PDGF and do not respond to it in biological assays [71], so presumably they would not be affected by the production of PDGF A chain by the tumor fibroblasts. Basic FGF has been shown to stimulate mitogenesis in MCF-7 and T47 D breast tumor cells [8], as well as a murine breast tumor cell line [72]. However, the overall growth regulatory effects on tumor epithelium of the fibroblast growth factors is less well defined than for the other families of growth factors analyzed here. Stromal FGF production may be important, however, in the stimulation of angiogenesis necessary for continued tumor growth.

It is important to note that the fibroblasts we studied are not transformed cells in that they show normal phenotypic behavior in tissue culture, with normal contact inhibition, monolayer growth, and senescence after approximately 15–20 passages. The cells are not monoclonal. Each line was pooled from all of the fibroblasts that grew from the original tumor. Therefore the

differences in IGF I and II expression seen between fibroblasts derived from malignant and benign lesions is not the result of a transformation event within those cells. Several explanations are possible for the difference in IGF-I and IGF-II gene expression described. First, the difference may have existed prior to tumor formation. If this is the case, it is conceivable that IGF-II expression in breast tumor stroma in some way predisposes to malignant transformation. Alternatively, it may be a passive marker of some other transforming event.

If the pattern of IGF expression we have demonstrated follows rather than precedes tumorigenesis, it may result from the recruitment and expansion of a preexisting stromal cell population. Less likely, there may be a change in the pattern of IGF gene expression in the stroma that results from interactions with transformed tumor epithelium. Since the pattern of IGF expression persists in serial culture in the absence of tumor epithelial cells, the mechanisms responsible for those stromal/epithelial interactions are not clear. We do not yet know whether the pattern of growth factor expression seen in the breast fibroblasts we have examined is the same for fibroblasts from skin or other organs in the same patients. At present we are examining growth factor expression in normal skin fibroblasts from patients with and without breast cancer, as well as fibroblasts from cancer families.

Having established the pattern of growth factor expression in breast fibroblasts, we then performed some preliminary experiments to ask whether paracrine interactions between different cell types can play a role in the regulation of growth factor expression by either breast tumor stromal or epithelial cells. We first asked whether PDGF, which is produced by breast cancer epithelial cells, could increase IGF-II mRNA expression in breast tumor fibroblasts.

Fibroblasts derived from a breast cancer were plated at equal density and allowed to grow in normal medium until the fibroblasts reached approximately 90% confluence. The fibroblasts were then switched to serum-free medium for an additional 48 hours. The cells were then treated with 10 ng/ml human recombinant PDGF BB homodimer. PDGF-treated fibroblasts were harvested and analyzed for IGF-II mRNA expression as multiple time points.

A complementary experiment was performed to analyze the effect of IGF treatment on the expression of PDGF mRNA by breast cancer epithelial cells. Since we had demonstrated IGF expression in breast fibroblasts, we asked whether IGF from these cells could increase PDGF expression in breast tumor epithelial cells. For this analysis, MCF-7 cells were grown in serum-free medium as described for the fibroblast experiment. They were then stimulated with either 40 ng/ml human recombinant IGF-I or 80 ng/ml human recombinant IGF-II. The cells were harvested and analyzed for PDGF A and B chain mRNA expression.

Breast tumor fibroblasts showed a threefold increase in IGF-II mRNA following treatment with human recombinant PDGF BB homodimer. The

induction was maximal after approximately 6 hours and declined to baseline from that point. Conversely, MCF-7 cells treated with insulin-like growth factor showed a twofold increase in PDGF-A chain mRNA after 6 hours of IGF treatment. There was a slight increase in PDGF-B chain mRNA as well.

While the changes in growth factor mRNA expression in these experiments are modest, they do suggest that cooperative intercellular communication between cell populations within a breast tumor can indeed take place. Some of these signals appear to be mediated by paracrine effects of soluble growth factors. The data discussed here raise the interesting possibility of cooperative paracrine effects between breast tumor stroma and epithelium. In the example presented here, we examined a model for interactions between PDGF and IGF. Despite the fact that breast-tumor epithelial cells produce both PDGF A and B chains, they have not been found to have PDGF receptors, and so presumably cannot respond to PDGF in an autocrine fashion. The observation that PDGF can induce an increase in IGF-II mRNA expression by stromal fibroblasts raises the possibility of an important paracrine role for PDGF production by tumor epithelial cells. Not only can PDGF from tumor epithelium accelerate the growth of stromal fibroblasts, but it may also induce the production of IGF by the fibroblasts, which in turn can act as an epithelial mitogen. This notion is supported by the observations of Clemmons, who reported an increase in IGF protein production in a radioimmunoassay analysis of fibroblasts treated with PDGF [73]. The observation that IGF treatment induced a slight increase in PDGF mRNA expression by MCF-7 cells suggests a possible cooperative paracrine loop between tumor epithelium and stromal fibroblasts. Epithelial production of PDGF stimulates stromal production of IGF, which in turn stimulates greater epithelial production of PDGF, etc. Just how important such a mechanism might be in promoting actual tumor growth is still speculative. A proposed model of these interactions is shown in Figure 1.

## 7. Conclusions

Stromal-epithelial interactions are critical determinants of growth and function in normal and malignant breast tissue. In breast cancer, overall tumor growth and spread represents a complex bidirectional balance between malignant tumor epithelium and its stromal microenvironment.

Studies of skin and breast stromal tissues in cancer patients are normals suggest that there are significant phenotypic differences in the stromal cell populations of these two groups. It remains to be more fully defined, however, whether these differences are pathophysiologically significant in tumor growth or whether they are incidental markers of malignancy. It also remains to be seen whether the phenotypic differences between stromal cells

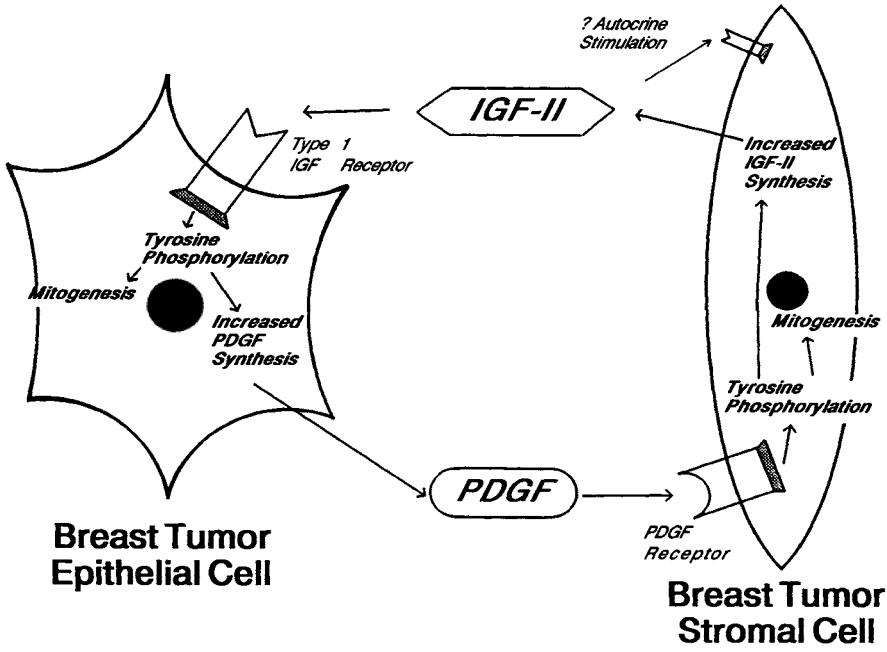


*Figure 1.* Proposed model for growth factor mediated stromal/epithelial interactions in breast cancer. IGF-II produced by breast tumor stromal cells binds to the type I IGF receptor on breast tumor epithelial cells. This results in a mitogenic signal for the epithelial cell, but also induces PDGF mRNA expression by the epithelial cell. PDGF in turn serves as a paracrine mitogenic signal for tumor stromal cells. In addition, PDGF induces IGF-II mRNA expression by the epithelial cell, completing a cooperative paracrine loop.

from benign and malignant breast tissue precede (and perhaps predispose to) or follow the carcinogenic event.

In vitro studies of stromal-epithelial interactions suggest that peptide growth factors may be responsible for significant intercellular signals that impact on overall tumor growth. The proposed model described in Figure 1 suggests that growth factors in this system may be functioning not simply as mitogens but as bidirectional signaling molecules as well. Better understanding of the role of growth factors in this setting may open new avenues for tumor therapy.

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