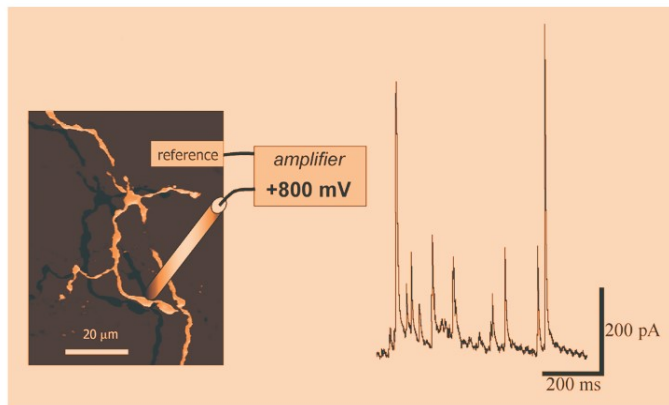


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# CARDIOVASCULAR GENOMICS

# CONTEMPORARY CARDIOLOGY

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# PREFACE

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With the postgenomic transcriptional era currently flourishing, the time seemed right to put together a book entitled *Cardiovascular Genomics*. Cardiovascular genomics is the study of genes relevant to the function and dysfunction of vital organs that both form and control the cardiovascular system. The book is organized into three parts: Part I: Genes and Polymorphisms in Cardiovascular Disease, Part II: Gene Transfer for Combating Cardiovascular Disease, and Part III: Regenerative Tissues for the Diseased Cardiovascular System.

*Cardiovascular Genomics* provides an up-to-date account of the most recent molecular approaches adopted to understand the cardiovascular system in both health and disease. The book provides an excellent resource for students, researchers, and clinicians on the potential of development of novel strategies for the control of cardiovascular diseases. The contributors are world leaders and the subject matter stretches from basic science to clinical applications, focusing on all components of the cardiovascular system—including vessels, heart, kidney, and the brain—and covers disease states ranging from vascular and cardiac dysfunction to stroke and hypertension.

Primary hypertension, a disease of the cardiovascular system, is a polygenic disease that has reached epidemic proportions worldwide. Most evidence suggests that susceptibility is genetically linked. Despite more than 50 years of using a large arsenal of antihypertensive agents, successful control of blood pressure is difficult to achieve. We believe this indicates that the time is ripe to explore alternate strategies. Cardiovascular genomics is all about doing just that. Discovering the genes that cause susceptibility to cardiovascular diseases will significantly increase the efficacy of treatment for these diseases. Remarkably, little is known about the numbers of genes involved in hypertension, what they do, their interactions with themselves and with others, and how they are modulated by stressors and diet, for example. *Cardiovascular Genomics* attempts to address these issues by focusing on both the villains and the victims.

Methods for identifying the genes that relate to a cardiovascular disease are discussed in relation to the possibility of discovering new drug targets. These approaches include discussions of genetic linkage analysis substitution mapping using congenic strains as well as microarray techniques. All are discussed as plausible strategies for discovery in animal models of hypertension. Not surprisingly, overactivity of the renin–angiotensin system may be one of the most common ailments and polymorphisms of angiotensin type I receptors and angiotensin-converting enzyme could well cause an overactivity of the signaling system. The new transgenic mouse models that either over- or underexpress angiotensinogen discussed within may help in addressing the important issue of the function of the tissue renin angiotensin systems. But an understanding of polymorphisms may also be crucial for the effective design of new drugs as well as successful pharmacotherapy for different patients; these important issues are thoroughly discussed, including those relating to statins.

A number of chapters in this book are dedicated to the theme of gene transfer and gene therapy covering a wide range of topics from clinical applications to using viral vectors as tools. For clinical applications, these include novel approaches to stroke, as well as

coronary and peripheral vascular diseases. The efficacy of gene therapy for safe gene delivery in cerebrovascular diseases has been used to stimulate angiogenesis, overexpression of vasodilator agents, or breakdown thrombi and stabilize plaques. These forms of somatic gene transfer have also been used to prevent both restenosis and vascular graft failure. Clear evidence has emerged that enhancing nitric oxide production reduces both oxidative stress and inflammatory responses that assist in lowering BP but also, independently, are beneficial in protecting against cardiac remodeling, renal fibrosis, restenosis and cerebral infarction as exemplified by adeno-associated viral-induced expression of kallikrein. In animal models of cardiovascular disease there are a number of chapters detailing a rapid expansion of viral vector technology in basic science. This includes applications to the heart, vasculature, and brain. The new strategies of virally mediated gene transfer include the design and effective use of expressing dominant negative proteins, siRNA, the employment of cell-specific promoters, and the use of vigilant vectors containing a physiologically operated genetic switch.

*Cardiovascular Genomics* also contains chapters on regenerative medicine that illustrate how molecular biology is enhancing the production of cardiovascular cells and tissues (e.g., cardiomyocytes and endothelial cells) from embryonic stem cells for repairing a diseased system. It is now clear that transplantation of genetically modified noncontractile cells into ischemic or dilated cardiomyopathic hearts can stimulate cardiomyogenesis that increases cardiac function. Moreover, regenerative medicine approaches now using ex vivo-engineered endothelial progenitor cells to treat peripheral and myocardial ischemia and genetically modified vein grafts have also proven successful.

The chapters in this book provide ample evidence for the considerable benefits that come from the application of genomic information and technologies to an understanding of both fundamental physiological processes and design of better therapies for one of the biggest killers worldwide. However, it is clear that many difficult challenges still lie ahead and must be confronted. Many methodologies and concepts are still evolving, common standards have yet to be established, and various problems with experimental design, variability, and statistical analysis still have to be fully understood and overcome. To fully realize the promise of the genomic approach, it is imperative that both scientists and physicians representing different disciplines work together to enhance progress. Only by means of multidisciplinary experimental approaches and the sharing of ideas can we develop novel cardiovascular therapeutics. Collaboration is the key that will unlock the genome.

The editors extend thanks to Ms. Nichole Herring for valuable assistance in putting together this volume.

*Mohan K. Raizada, PhD*  
*Julian F. R. Paton, PhD*  
*Sergey Kasparov, MD, PhD*  
*Michael J. Katovich, PhD*

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# I

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## GENES AND POLYMORPHISMS IN CARDIOVASCULAR DISEASE

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# 1

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## Angiotensin II Receptor Polymorphisms and Hypertension

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*Bruno Baudin*

### CONTENTS

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### SUMMARY

Molecular variants of individual components of the renin–angiotensin system have been thought to contribute to inherited predisposition toward essential hypertension. Angiotensin II type 1 receptor (AT-1) mediates the major pressor and trophic actions of angiotensin II (Ang II). Moreover, polymorphisms in genes of angiotensinogen and angiotensin-converting enzyme (ACE) have been associated with arterial hypertension and cardiovascular diseases, and some of them have been related to differential responses to antihypertensive drugs. So far, at least 25 different polymorphisms have been described in *AT-1* gene (*AT<sub>1</sub>R* gene), both at the 3' untranslated region and in its promoter region. Best evaluated with respect to the association with cardiovascular phenotypes is the +1166 A/C polymorphism. In particular, the C allele has been associated with the severe form of essential hypertension and in some studies an association was found between C allele in *AT<sub>1</sub>R* gene and D allele in ACE gene; but large discrepancies arise from ethnic variability. The role of *AT<sub>1</sub>R* A1166C polymorphism is ambiguous in pathologies related to high Ang II levels, such as deterioration of renal function (for example in diabetes), arterial stiffness, and hypertrophic cardiomyopathy. Recently, polymorphisms have also been described in angiotensin II type 2 receptor (AT-2) gene (*AT<sub>2</sub>R* gene), AT-2 being the mediator for vasodilatation, natriuresis, and apoptosis of smooth muscle cells. Associations were found between some of these polymorphisms and left ventricular structure, whereas the response to Ang II infusion did not differ across *AT<sub>1</sub>R* and

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AT<sub>2</sub>R genotypes. On the other hand, a relationship was suggested between AT<sub>1</sub>R A1166C polymorphism and the humoral and renal hemodynamic responses to losartan, an antihypertensive drug acting as an AT-1 blocker, as well as with enhanced Ang II vascular reactivity or sensitivity even when conflicting results were observed. The variability in the individual response to AT-1 antagonists could also result from variations in the pharmacokinetics of the drugs; in particular, losartan is essentially metabolized to its active form by cytochrome P450 2C9, which biotransforms many cardiovascular drugs but at different rates in function of both ethnic and individual genotypes. The other angiotensin II receptor, AT-2, should also be investigated because the different AT-1 antagonists do not share the same selectivity for both subtypes but all are able to increase Ang II levels, which enhances AT-2 related effects. Arterial hypertension is one of the main risk factors for stroke and coronary artery disease (CAD); however, no clear association was found between AT<sub>1</sub>R gene polymorphisms and the development of white matter lesions, stroke, CAD, or myocardial infarction, although some studies described relationships between AT<sub>1</sub>R A1166C polymorphism and hypercholesterolemia, or greater induced arterial vasoconstriction in CAD. Moreover, AT<sub>1</sub>R C allele, when associated with ACE D allele (of I/D polymorphism), could contribute to susceptibility to CAD and to interindividual differences in severity of cardiovascular disease. Further evaluation in adequately powered studies is necessary for final assessment of allelic markers in RAS component genes, namely *AT<sub>1</sub>R* and *AT<sub>2</sub>R* genes, as well as to determine predisposition to hypertension or related diseases, and to choose an antihypertensive drug for an individual and to develop more specifically targeted drugs.

**Key Words:** Renin–angiotensin system; angiotensin II receptors; single nucleotide polymorphism (SNP); antihypertensive therapy; angiotensin II receptor antagonists; cardiovascular disease.

## INTRODUCTION

High blood pressure (BP) is an important risk factor for cardiovascular diseases, kidney failure, and stroke. It is recognized as a multifactorial trait resulting from a combination of environmental and genetic factors. The genetic influences (or heritability) were examined in both cross-sectional samples of data and longitudinal studies. Efforts to date have identified several candidate genes involved in blood pressure or primary hypertension. Special attention has been paid to the study of genes implicated in the renin–angiotensin system (RAS) because its activation and the subsequent generation of angiotensin II (Ang II) both play important roles in normal physiology and in the progression of cardiac and renal diseases. Most of the known actions of Ang II are mediated by the Ang II type 1 receptor (AT-1), including vascular contraction, pressor responses, renal tubular sodium transport and aldosterone secretion. Antagonists of the AT-1 have been developed and are now widely used in the treatment of hypertension (*1*). Recently, a polymorphism in AT-1 gene (*AT<sub>1</sub>R* gene) has been related to arterial hypertension and other cardiovascular impairments. Moreover, a relationship was suggested between this polymorphism and the humoral and renal hemodynamic responses to losartan, the first AT-1 blocker. This chapter will present the actual knowledge of the Ang II receptors and the polymorphisms in their genes related to hypertension, and others related to cardiovascular diseases. We will also discuss these data from the area of pharmacogenomics, including variations in the pharmacokinetics of the antihypertensive drugs.

## THE ANGIOTENSIN II RECEPTORS

The RAS is a major physiological regulator of body fluid volume, electrolyte balance, and arterial pressure. Originally, the RAS was viewed solely as an endocrine system;



angiotensinogen of hepatic origin is secreted into the systemic circulation and cleaved to Ang I by renin (in its active form as found in blood plasma), and then by both plasma and endothelial membrane-bound ACE to produce the active octapeptide Ang II. Increasing evidence suggests that other complete RAS may reside within individual organs or tissues such as kidney, lung, heart, and vascular smooth muscle cells, where they could act in a functionally independent paracrine/autocrine fashion (2,3).

Ang II is the major effector peptide of this system and the biological actions of Ang II are mediated by at least two different receptor subtypes, AT-1 and AT-2. Because AT-2 has a low degree of expression compared with that of AT-1, virtually all of the biological actions of Ang II, as well as its paracrine and autocrine regulations including cell growth, proliferation, and extracellular matrix formation have been attributed to its binding to AT-1 (4). Both Ang II receptors have been cloned and their signal transduction mechanisms have been clarified. We have now gained a great deal of knowledge concerning the receptor genes, molecular and protein structures, sites, and regulation of expression and mechanisms of action. The gene coding for AT-1 (*AT<sub>1</sub>R* gene) is located on human chromosome 3 (q22 band), whereas the gene coding for AT-2 (*AT<sub>2</sub>R* gene) is located on chromosome X (q23-24 locus). AT-2 is only 34% identical with its AT-1 counterpart. Both receptors belong to the G protein-coupled receptor superfamily containing seven hydrophobic transmembrane segments forming  $\alpha$  helices in the lipid bilayer of the cell membrane. The binding of Ang II to the agonist site of the AT-1 receptor induces a conformational change in the receptor molecule that promotes its interaction with G proteins, which in turn mediate signal transduction via the phospholipases C, D, A<sub>2</sub>, and adenylyl cyclase, followed mainly by phosphoinositide hydrolysis and Ca<sup>2+</sup> signaling (4–6). Based on imidazole derivatives first described by Furukama et al. (*patent*), it became possible to develop specific nonpeptide Ang II receptor antagonists that specifically and selectively block AT-1. The first of this series to reach the clinic, losartan, was followed by a large number of orally active AT-1 antagonists (1). Blockade of AT-1 not only inhibits smooth muscle contraction, but also reduces the production of pressor agents including aldosterone, vasopressin, catecholamines, and endothelin. The AT-1 antagonists are approved for the treatment of hypertension, and in early clinical studies also appear to be of use in the treatment of congestive heart failure, postmyocardial infarction, and renal failure (7). Moreover, treatment with AT-1 antagonists has been found to abolish the growth-promoting actions of Ang II in vitro and in vivo (1,4).

AT-2 is clearly distinct from AT-1 in tissue-specific expression, signaling mechanisms, and diversity in molecular weight. Almost complete divergence between AT-1 and AT-2 is seen in the third intracellular loop, and more extensive differences in the carboxyl terminal tail. The structural diversity of AT-2 is still a complex and unresolved issue, even when oligomerization and differences in the extent of glycosylation are involved. In contrast to the hypertension and impaired vascular responses observed in AT-1-deficient mice (knockout mice for *AT<sub>1</sub>R* gene), knockout of the AT-2 leads to elevation of BP and increased vascular sensitivity to Ang II; it stimulates vasodilatation and natriuresis by an autocrine cascade including bradykinin, nitric oxide, and cyclic GMP. This has suggested that AT-2 may exert a protective action in BP regulation by counteracting AT-1. Moreover, whereas the AT-1 stimulates cell proliferation, AT-2 inhibits proliferation and promotes cell differentiation. These differences in growth responses have been ascribed to different cell signalling pathways in which AT-1 stimulates protein phosphorylation and AT-2 stimulates dephosphorylation. In particular, AT-2 could negatively modulate the AT-1-mediated activation of both phospholipase C (for

control of vascular tone and natriuresis) and MAPkinase (for control of cell growth). Moreover, AT-2 is able to induce apoptosis and may play a role in cognitive functions and certain types of behavior. By inhibiting the AT-1-mediated negative feedback of Ang II on renin release in the kidney, AT-1 antagonists evoke an overstimulation of the uninhibited AT-2 by enhancing Ang II levels in plasma. However, because the exact physiological roles of AT-2 are not well defined, it is uncertain as to whether this overstimulation of AT-2 would produce beneficial or harmful side effects (4,8).

Recently AT-4, a new angiotensin receptor type, that preferentially binds Ang II (3-8) (a fragment of Ang II, now referred to as Ang IV) has been discovered and characterized. This receptor is prominent among brain structures concerned with cognitive processing and motor and sensory functions. Like AT-2, it may also oppose the effect of AT-1 as it regulates blood flow, inhibits tubular sodium reabsorption, and affects cardiac hypertrophy (4).

In conclusion, most of the known effects of Ang II are mediated through AT-1, including vasoconstriction, aldosterone and vasopressin release, salt and water retention and sympathetic activation, in addition to the important autocrine and paracrine effects of Ang II on cell proliferation and tissue remodeling. Accumulated published data suggest that AT-2 and AT-4 counterbalance the effects of AT-1 in both physiological and pathophysiological situations.

### SNP IN *AT<sub>1</sub>R* GENE

Polymorphisms (genetic variations of high frequency) may be the result of variations in the DNA sequence, such as the change of only one nucleotide (single nucleotide polymorphism [SNP]), or an insertion or deletion of one to several hundred bases resulting from the DNA recombination failures, or insertion/deletion of DNA repeating sequences, such as tandem repeat sequences, microsatellites, or AluI sequences. The human *AT<sub>1</sub>R* gene has a length of greater than 55 kb and is composed of five exons and four introns, and has been found highly polymorphic. In particular, an SNP has been described in which there is either an adenine (A) or a cytosine (C) base (A/C transversion) in position 1166 in the 3' untranslated region of the gene (9); until now this +1166 A/C polymorphism was the best evaluated. The A allele that lacks the enzyme-restriction site is designated as the larger fragment, whereas the C allele that has an enzyme-restriction site at nucleotide position 1166 is designated as the smaller fragment. The physiological significance of this polymorphism is uncertain because of its location in an untranslated region. Another SNP at nucleotide position +573 was investigated in hypertension and diabetes (10,11). Erdmann et al. (12) characterized nine other SNPs, which may have the potential to influence *AT<sub>1</sub>R* gene expression given their location in the functional promoter region of the gene; these variants were found to be strongly linked, such that the analysis of three SNPs should provide information for all possible haplotypes, i.e., -2228 G/A, -1424 C/G, and -521 C/T. Two other groups found many other SNPs in the 5'-flanking region of the gene: Poirier et al. (13) detected seven polymorphisms, i.e., at -1424, -810, -713, -521, -214, -213, and -153 positions, not in linkage equilibrium with A1166C polymorphism, and some of them having also be found by Erdmann et al. (12). Takahashi et al. (14) identified seven other polymorphisms, i.e., at -1154, -825, -729, -535, -227, -226, and -166 positions; these polymorphisms also do not occur at random, leading to the classification into three haplotypes. Recently, Zhu et al. (15) described five other SNPs at both 5'- and 3'-flanking regions, i.e., -777, -680, -119, +43732, and +44221.

## AT<sub>1</sub>R GENE POLYMORPHISMS AND HYPERTENSION

Hypertension has a multifactorial etiology with a strong genetic component. Of many human candidate gene loci examined, those encoding components of the RAS are considered to be among the most plausible candidates. The association of *renin* gene polymorphism with essential hypertension has been scarcely reported (16). On the contrary, several studies have demonstrated a link between the *angiotensinogen* (*AG*) gene and hypertension (17–19), particularly the M235T variant which correlates with both plasma angiotensinogen concentration and elevated BP variation; nevertheless, large ethnic discrepancies make this genotyping difficult to use.

The cloning of *ACE* gene enabled the detection of many polymorphisms (20,21), one of which consists of the presence (insertion allele I) or absence (deletion allele D) of a 287-bp DNA fragment in intron 16 of the *ACE* gene. A strong association was detected between the alleles of this polymorphism and the level of serum ACE, individuals homozygous for the D allele (genotype DD) displaying serum ACE levels almost twice as high as those in individuals homozygous for the I allele (genotype II) but without clear correlation to BP (20,22,23). In particular, no association was found between this I/D polymorphism in the *ACE* gene and essential hypertension in young, middle-aged, or elderly populations, even when interindividual variation in BP could be dependent on contexts that are indexed by gender, age, and measures at body size. One possible explanation is that this polymorphism is in linkage disequilibrium with functional variants elsewhere in the *ACE* gene that are responsible for the BP.

For *AT<sub>1</sub>R* gene, the silent A1166C SNP has been associated with the severe form of essential hypertension, and in particular in resistant hypertensive patients taking two or more antihypertensive drugs (9,24). The C allele was particularly overrepresented in Caucasian hypertensive subjects with a strong family history (25), and it was also significantly more frequent in women with pregnancy-induced hypertension, whereas *ACE* I/D and *AG* M235T polymorphisms were not associated with predisposition to development of hypertension in pregnant women (26). However, a significant interaction between the *ACE* I/D and *AT<sub>1</sub>R* A1166C polymorphisms in terms of influence on BP variation has been reported (27), but their linkage mechanism remains unclear. Henskens et al. (28) recently confirmed an association of both these polymorphisms with BP in healthy normotensive subjects, although synergistic effects did not seem to be present; in these studies *ACE* D allele and *AT<sub>1</sub>R* C allele were associated with highest pressures, either systolic, diastolic, or both. Berge et al. (29) also found an association of *AT<sub>1</sub>R* C allele with systolic BP in normotensive subjects. But large interethnic differences in the frequencies of genotype polymorphisms of the RAS exist; for example, the *ACE* DD and the *AT<sub>1</sub>R* CC genotypes have low prevalence in Chinese subjects compared with whites, whereas the *AG* M235T variant is much higher in Asian populations compared with white populations. Moreover, a higher prevalence of the *AT<sub>1</sub>R* CC genotype was found in Chinese hypertensive patients than in controls (30), whereas the A1166C genotype distribution did not differ between hypertensive and normotensive subjects from Japan (31) or Taiwan (32). In a sample of Swedish twins, Iliadou et al. (33) did not find any significant linkage and association between *ACE* I/D polymorphism or *AT<sub>1</sub>R* A1166C polymorphism and BP. In Caucasoid subjects from Germany, Schmidt et al. (34) did not detect any association of A1166C polymorphism with hypertension, but a trend towards a decreased prevalence of the C allele among hypertensive patients with a late age at diagnosis (>50 yr) was observed. Tiret et al. (35) showed a higher prevalence of C allele

among female hypertensives than in controls but no such difference was observed for men. Szombathy et al. (36) did not find any difference for this polymorphism in *AT<sub>1</sub>R* gene between normotensive controls and subjects with resistant essential hypertension, but high values of systolic BP were associated with the C allele in older and overweight patients. Moreover, Castellano et al. (37) found that BP values were lower in CC homozygotes in an Italian population; these homozygotes also presented a lower incidence of family history of hypertension.

The molecular basis of salt sensitivity in human hypertension was explored by De la Sierra's team, and in particular by the evaluation of *RAS* gene polymorphisms; patients with *ACE* II or ID genotype had significantly higher prevalence of salt sensitivity than DD hypertensives, whereas *AT<sub>1</sub>R* A1166C polymorphism had no effect (38,39). Moreover, Tabara et al. (40) did not find any significant associations between orthostatic hypotension and gene polymorphisms of *ACE* I/D, *AG* M235T, and *AT<sub>1</sub>R* A1166C, orthostatic hypotension being an important risk factor for future cardiovascular morbidity and mortality. Several studies explored the sensitivity to Ang II in relation to *AT<sub>1</sub>R* A1166C polymorphism; for example, in normotensive subjects, Paillard et al. (41) did not find any genotype effect of this polymorphism on the B<sub>max</sub> and K<sub>D</sub> values of AT-1 on platelets. Testing for the response to short term infusion of Ang II in normotensive and moderately hypertensive subjects, Hilgers et al. (42) also concluded that the A1166C polymorphism does not have a major effect on the action of Ang II, i.e., increases in BP, plasma aldosterone, glomerular filtration rate, and decrease in renal blood flow.

Hypertension is a major risk factor for stroke, renal failure, and cardiovascular diseases. *RAS* gene polymorphism was studied in patients with cerebral white matter lesions assessed by brain magnetic resonance imaging; these lesions represent a subclinical form of ischemic brain damage and hypertension is one of the most important factors for their development. Sierra et al. (43) showed that the presence of the *ACE* D allele may be a predisposing factor for developing white matter lesions in essential hypertensive patients, whereas no significant association for the *AG* M235T and *AT<sub>1</sub>R* A1166C polymorphisms was found. Moreover, no association was shown between *AT<sub>1</sub>R* gene polymorphisms and stroke (35). On the contrary, the presence of the C allele in *AT<sub>1</sub>R* gene might be associated with faster deterioration of renal function, i.e., progression to end-stage renal disease, and whatever the aetiology of the renal failure (44,45). Originally, a synergistic effect was suggested for *AT<sub>1</sub>R* A1166C polymorphism and poor glycemic control on the risk of diabetic nephropathy in insulin-dependent diabetic patients (11). But the subsequent studies performed failed to find any association between this polymorphism in *AT<sub>1</sub>R* gene and diabetic nephropathy (46–50). *RAS* gene polymorphism was also investigated in obesity, and particularly in obesity-associated hypertension. No association was detected between *AG* M235T or *AT<sub>1</sub>R* A1166C polymorphism and anthropometric indexes or BP, whereas the *ACE* I/D polymorphism was a significant predictor of overweight and abdominal adiposity in man, the DD homozygosity being associated with larger increases in body weight and BP in aging persons, as well as with higher incidence of overweight (51).

Arterial stiffness is associated with excess morbidity and mortality, independently of other cardiovascular risk factors. Age is the main determinant responsible for arterial wall changes leading to arterial stiffening. However, arterial stiffness is also influenced by vasoactive systems, and particularly the *RAS*. When *AG* M235T, *ACE* I/D, and *aldosterone synthase* gene polymorphisms were not associated with aortic stiffness as assessed by carotid-femoral pulse wave velocity, the 1166C allele in *AT<sub>1</sub>R* gene influenced the relationship between age and arterial pulse wave velocity and in an additive effect with

another SNP in *AT<sub>1</sub>R* gene, the -153 A/G (52). The C allele was also associated with aortic stiffness in both normotensive and hypertensive subjects (53), and moreover in correlation with the ratio of total cholesterol on high-density lipoprotein cholesterol (54). Conversely, Girerd et al. (55) did not find such a correlation with vascular hypertrophy as assessed by high resolution echography in subjects with no evidence of cardiovascular disease.

Hypertrophic cardiomyopathy occurs as a familial disorder with at least six genes clearly identified; but other factors, genetic as well as environmental, may modify the phenotypic expression of the mutated gene. Ang II is an important modulator of cardiac hypertrophy, and ACE inhibition induces regression of cardiac hypertrophy and prevents dilatation and remodelling of the ventricle after myocardial infarction. Osterop et al. (56) investigated whether the *ACE* I/D and *AT<sub>1</sub>R* A1166C polymorphisms influence left ventricular hypertrophy in subjects with hypertrophic cardiomyopathy and concluded that the C allele in *AT<sub>1</sub>R* gene modulates the phenotype of hypertrophy as determined by two-dimensional echocardiography, and independently of *ACE* I/D polymorphism. Takami et al. (57) also reported an association between C allele and left ventricular mass index, but in normotensive subjects without hypertrophic cardiomyopathy. These results are not in accordance with the study of Hamon et al. (58), which does not find any association of the *AT<sub>1</sub>R* A1166C polymorphism with left ventricular hypertrophy in subjects with normal coronary arteries; however, a strong association was observed between left ventricular mass and systemic hypertension. Ishanov et al. (59) also concluded that this polymorphism does not contribute to the development of cardiac hypertrophy in patients with hypertensive left ventricular hypertrophy or hypertrophic cardiomyopathy. Moreover, no kind of relationship between AT-1 density and/or function and *AT<sub>1</sub>R* gene C allele has yet been observed (60). Investigating patients by echocardiography for idiopathic heart failure diagnosis, Andersson et al. (61) found that patients with *ACE* DD and *AT<sub>1</sub>R* CC or AC genotypes tended to have lower ejection fraction and increased left ventricular mass, suggesting an interaction of these two genetic traits on disease severity. Hamon et al. (58) also observed that the subjects homozygous for the *AT<sub>1</sub>R* CC genotype had a significantly lower ejection fraction than those with A allele. These studies appear to suggest that in severe forms of hypertension, the *AT<sub>1</sub>R* A1166C SNP is potentially involved in the regulation of BP; even when contrasted with other components of RAS, no direct effect of this polymorphism on the gene product has been demonstrated until now. Moreover, results from one ethnic group cannot be extrapolated to another.

Among the other polymorphisms in *AT<sub>1</sub>R* gene, at 5'-flanking region a higher frequency of the T allele (-535C/T SNP) was observed in hypertensive patients (14), whereas Zhang et al. (62), evaluating nine newly characterized SNPs, did not show any association with arterial hypertension. Poirier et al. (13) also noticed that among seven new polymorphisms, all different from those formerly described, none was associated with pressor levels in control subjects, whereas Chaves et al. (10) found that the +573C/T polymorphism might be a genetic protective factor for urinary albumin excretion in a population of essential hypertensives. Investigating 25 new polymorphisms in RAS genes, Zhu et al. (15) particularly described an association between two *AT<sub>1</sub>R* gene polymorphisms, located at the 3'-flanking region, and hypertension in African Americans but not in European Americans.

Recently, polymorphism in *Ang II type 2 receptor (AT<sub>2</sub>R)* gene was also shown and investigated, in particular the A3123C located in the 3' untranslated region of exon 3 in a study showing that this polymorphism may contribute to cardiac hypertrophy in women with hypertrophic cardiomyopathy (63). Conversely, no association was detected between

*AT<sub>2</sub>R* polymorphism and hypertension (57). Delles et al. (64) tested another SNP in *AT<sub>2</sub>R* gene, namely +1675 G/A polymorphism, and in parallel of a SNP in *AT<sub>1</sub>R* gene, i.e., -2228 G/A polymorphism; the response to Ang II infusion did not differ across the *AT<sub>1</sub>R* and *AT<sub>2</sub>R* genotypes. Conversely, this latter polymorphism in *AT<sub>2</sub>R* gene was associated with left ventricular structure in young adults with normal or mildly elevated BP; in particular, as stated by echocardiography, hypertensive subjects with the A allele had greater left ventricular thickness and mass index than those with the G allele, suggesting early structural changes of the heart owing to hypertension in association with a genetic trait in RAS (65). Research on AT-2 has unveiled hitherto unknown functions of the RAS extending beyond the classical role of this hormonal system in cardiovascular control, providing a solid basis for further research variation (66), and in particular using AT-1 antagonists.

### *AT<sub>1</sub>R* GENE POLYMORPHISM AND ATHEROSCLEROSIS-RELATED DISEASES

Coronary artery disease (CAD) is a major public health problem in industrialized countries. Dyslipidemia, arterial hypertension, and diabetes mellitus, the main risk factors for CAD apart tobacco consumption, are influenced by both environmental and genetic factors. Several studies have suggested that the activation of the RAS could be an important contributor to CAD. The best-documented of associations between the occurrence of CAD and polymorphisms in genes RAS component is the I/D polymorphism of the *ACE* gene. After the initial work of Cambien et al. (67), who first suggested the potential role of the *ACE* D allele as an independent risk factor for myocardial infarction (MI), other case-control studies either confirmed or were in disagreement with their findings (68–70). The M235T variant of *AG* gene has also been associated with MI but, as in hypertension, with ethnic variability (19). A1166C polymorphism in *AT<sub>1</sub>R* gene does not seem to be a direct risk factor for CAD or MI, but, in many studies, it has been observed to have synergistic effects with *ACE* I/D polymorphism (53,71–73); in particular the highest risks were shown for patients with both *ACE* DD and *AT<sub>1</sub>R* CC genotypes. Conversely to the former studies, other examinations did not find an association between A1166C polymorphism in *AT<sub>1</sub>R* gene and CAD or MI, as well as no interaction between this polymorphism and I/D polymorphism in *ACE* gene (74–76) or independently of BP (77). Moreover, Olivieri et al. (78) did not find associations between *AG* M235T or *AT<sub>1</sub>R* A1166C polymorphism and atheromatous renal artery stenosis, whereas their results suggested a predisposing role for *ACE* D allele in the development and progression of this atherosclerosis related disease. *AT<sub>1</sub>R* A1166C polymorphism was also not related to hypercholesterolemia, in particular in subjects with essential hypertension (79); but in another study C allele was overrepresented in familial hypercholesterolemia with CAD (80). An association was found between *AT<sub>1</sub>R* C allele and *ACE* D allele in patients with CAD and malignant ventricular arrhythmia treated with an implantable cardioverter defibrillator (81). Recently, Gruchala et al. (82), evaluating left ventricular size and performance of patients with angiographically confirmed CAD by echocardiography, did not find relationships between *AT<sub>1</sub>R* A1166C polymorphism and left ventricular parameters. In two studies, patients carrying the *AT<sub>1</sub>R* CC genotype have greater vasoconstriction in coronary vessels, as well as in response to Ang II infusion (83), than after intravenous injection of methylethylergonovine maleate, a potent vasoconstrictor whose effects have been explored in CAD (84); but Steeds et al. (85) did not find differences in

vasomotor function in isolated human mesenteric resistance arterioles, but an increase in sensitivity to prostaglandin F<sub>2</sub> $\alpha$  in arterioles from patients with C allele in *AT<sub>1</sub>R* gene. Vuagnat et al. (86) found the same following Ang II infusion to assess familial resemblance in hypertensive patients. Ortlepp et al. (87) concluded that left ventricular hypertrophy in patients with aortic stenosis was not determined by five genetic polymorphisms in RAS, particularly in *ACE*, *AT<sub>1</sub>R*, and *AG* genes. In a large population-based cohort of older adults, Hindorff et al. (88) did not observe any association of this *AT<sub>1</sub>R* gene polymorphism with incident cardiovascular events. Thus, taken alone, the A1166C polymorphism in *AT<sub>1</sub>R* gene neither represents a risk factor for adverse events complicating coronary interventions nor seems to have significant impact on further long-term processes such as development and severity of CAD (89–91). Recently, polymorphisms in *AT<sub>2</sub>R* gene were explored as predictors of CAD. Ye et al. (73) did not find any association between +1291 G/T or +1297 G/A polymorphism and coronary extent scores defined essentially by the number of coronary segments with significant stenosis. On the other hand, Jones et al. (77) demonstrated that hypertensive carriers with *AT<sub>2</sub>R* 1675A/3123A haplotype were at most risk for a cardiovascular event.

In conclusion, among these three well-documented polymorphisms in RAS, i.e., I/D polymorphism in the *ACE* gene, M235T variant of *AG*, and A1166C SNP in *AT<sub>1</sub>R* gene, only two types of relationships seem to be confirmed: (1) D allele of the *ACE* gene as risk factor for CAD and MI; and (2) C allele of A1166C in the *AT<sub>1</sub>R* gene, when associated with *ACE* D allele, as contributor to susceptibility of CAD and to interindividual differences in severity of cardiovascular disease.

## PHARMACOGENOMIC CONSIDERATIONS ON ANG II RECEPTORS

The response of patients to antihypertensive therapy is variable; individuals may respond differently to different medications, suggesting that treatment should be matched to individual responsiveness. The two main targets in RAS for an antihypertensive therapy are *ACE* and *AT-1*, with *ACE* inhibitors and *AT-1* antagonists (or blockers), respectively. Then, polymorphisms in *ACE* and *AT<sub>1</sub>R* genes may have influence on the antihypertensive response after, of course, *ACE* inhibitor or *AT-1* blocker treatment, but also after other antihypertensive drugs such as diuretics,  $\beta$ -blockers, calcium channel antagonists, or drugs acting on the central nervous system. For *ACE* I/D polymorphism, a recent review has assessed the first conclusions arising from pharmacogenomic studies (23). But few data are available for polymorphisms in *AT<sub>1</sub>R* gene (92). Miller et al. (93) hypothesized that renal and systemic Ang II activity would be augmented in subjects with C allele of *AT<sub>1</sub>R* gene A1116C polymorphism, and tested this hypothesis by comparing hemodynamic and humoral responses to *AT-1* blockade with losartan (the first *AT-1* antagonist) and with low-dose suppressor infusions of Ang II. In this study, glomerular filtration rate, renal plasma flow, and renal blood flow were significantly lower in subjects with C allele and during Ang II infusion; subjects with C allele maintained glomerular filtration rate despite equivalent declines in renal blood flow, suggesting an enhanced efferent arteriolar constrictive response. Losartan increased the glomerular filtration rate and decreased mean arterial pressure in this group of subjects; conversely, aldosterone responses to losartan were blunted in the AA subgroup. This study demonstrates an association between the C allele and the hemodynamic response to losartan treatment. Spiering et al. (94) also showed that hypertensive patients had decreased glomerular filtration rate and aldosterone but increased atrial natriuretic peptide. More-

over, Kurland et al. (95) showed that C allele is associated with a reduction in both endothelium-dependent and -independent vasodilations in normotensive individuals, whereas D allele in *ACE* gene was only reduced in endothelium-dependent vasodilatation. Nevertheless, these and the former authors did not discuss the interest in considering A1166C SNP in *AT<sub>1</sub>R* gene an important factor when deciding on antihypertensive therapy for individuals with primary hypertension. Several studies examined relationships between *ACE* and *AT<sub>1</sub>R* genes during antihypertensive therapy, but without AT-1 receptor antagonists. Dieguez-Lucena et al. (96) quantitated both the AT-1 and AT-2 messengers in peripheral blood mononuclear cells, and concluded that AT-1, but not AT-2, messenger level varies in relation to *ACE* I/D genotype, because AT-1 messenger and plasma Ang II levels were positively correlated with the D allele in the same individuals, and because AT-1 messenger levels decreased significantly with ACE inhibitor treatment in subjects with DD genotype. These effects were not seen with a  $\beta$ -blocker or a calcium channel antagonist in place of an ACE inhibitor, confirming close relationships between *ACE* and *AT<sub>1</sub>R* genes. Moreover, Benetos et al. (97) showed that according to the A1166C genotype in *AT<sub>1</sub>R* gene, an ACE inhibitor and a calcium channel antagonist affect pulse wave velocity (which evaluates aortic stiffness) but in opposite ways—i.e., baseline pulse wave velocity values were higher in subjects with C allele, and perindopril (an ACE inhibitor) decreased much more pulse wave velocity in these subjects, whereas nitrendipine (a calcium channel antagonist) decreased pulse wave velocity only in subjects with AA genotype. In another study, *ACE* and *AT<sub>1</sub>R* genotypes were not related to BP responses after ACE inhibition; only the AG M235T variant was an independent predictor of the BP response, with a fall in both systolic and diastolic pressures lowest in homozygotes for the M form compared with hypertensive subjects bearing one or two copies of the variant allele (98). Conversely, Dudley et al. (99) did not find any association between response to atenolol (a  $\beta$ -blocker), or lisinopril (an ACE inhibitor), or nifedipine (a calcium channel antagonist) and either the AG or *ACE* I/D polymorphisms; and Kurland et al. (100) did not show relationships between AG M235T or *AT<sub>1</sub>R* A1166C polymorphism and response for 3 mo to treatment with irbesartan (an AT-1 antagonist) or atenolol, whereas *ACE* I/D predicted the blood-lowering response to these antihypertensive therapies.

The variability in the individual response to AT-1 antagonists could also result from variations in the pharmacokinetics of the drugs; in particular, losartan is essentially metabolized to its active form by cytochrome P450 2C (*CYP2C9*), which biotransforms many cardiovascular drugs (16,101). *CYP2C9* activity varies markedly among different ethnic populations, particularly in relation to the frequencies of three alleles in *CYP2C9* gene, thus leading to different interethnic responsiveness to a given drug; this could also be the case for losartan (16). Interestingly, Uchida et al. (102) recently described the case of a man with a poor metabolizer genotype of *CYP2C9* who was hospitalized with severe hypotension 2 d after treatment of hypertension with candesartan, another AT-1 blocker. For other AT-1 blockers, such as irbesartan, other allelic variations could be involved for predicting antihypertensive response, but the enzymes responsible for their metabolism in humans have not been reported hitherto.

## CONCLUSIONS

Genetic heterogeneity is involved in the metabolism of cardiovascular drugs and the pathogenesis of inherited cardiovascular disorders. Molecular genetic technologies can



now provide efficient genetic testing, not only to identify disease-associated genes for diagnosis and risk stratification of many cardiovascular diseases, such as arterial hypertension, coronary artery disease, and diabetic nephropathy, but also to determine the responsiveness to a given drug, particularly in the area of antihypertensive therapy. Identification of the genes responsible for severe inherited forms of hypertension may provide novel diagnostic tools, as well as an opportunity for targeted therapeutic intervention. Candidate genes have been selected from the systems physiologically implicated in BP regulation, namely genes of the renin–angiotensin system as susceptible genes interacting with environmental factors for developing essential hypertension. *Renin*, *angiotensinogen*, *angiotensin I-converting enzyme*, and *angiotensin II receptor* genes have been evaluated, and emerging evidence has suggested that polymorphisms in both *AG* and *AT<sub>1</sub>R* genes are associated with certain forms of hypertension. Moreover, even when conflicting results were seen, polymorphisms in *AG* and *ACE* genes seem to be useful in the determination of the responsiveness to antihypertensive therapies, at least when using ACE inhibitors for the latter. For the polymorphisms detected in *AT<sub>1</sub>R* gene, and moreover in *AT<sub>2</sub>R* gene, the pharmacogenomic studies are still scarce, but interestingly, both *ACE* and *AT<sub>1</sub>R* genes loci could be associated. Clearly, further evaluation in adequately powered studies is necessary for final assessment. Perhaps when we are able to determine a few genotypes, such as some useful allelic marker SNPs in RAS genes, we might better choose an antihypertensive drug (or an association of drugs), maybe not positively but negatively, eliminating the worst and therefore making the choice easier among the remaining drugs.

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## Angiotensinogen Gene Polymorphisms and Hypertension

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*Ashok Kumar, PhD*

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## SUMMARY

Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure. Hypertension affects 50 million Americans with a prevalence rate of 25–30% in the adult Caucasian population. The incidence of hypertension and complications resulting from hypertension are even greater in the African-American population. The renin-angiotensin system plays an important role in the regulation of blood pressure, and previous studies have suggested that the angiotensinogen (*AGT*) gene locus is linked with human essential hypertension. Previous studies have suggested that a single nucleotide polymorphism that converts methionine to threonine at amino acid 235 is associated with hypertension in Caucasian population. This polymorphism is in linkage disequilibrium with A/G polymorphism at –6 position in the promoter of *AGT* gene. Reporter constructs containing variant A at –6 have increased promoter activity on transient transfection in human liver cells, suggesting that this variant may have increased transcriptional activity. However, this polymorphism is not associated with hypertension in the African-American and Chinese populations. We have found an A/G polymorphism at –217 of the human *AGT* gene promoter and have shown that frequency of allele A at –217 is significantly increased in the DNA of African-American hypertensive patients. We have also shown that: (a) reporter constructs containing *AG* gene promoter with nucleoside A at –217 have increased promoter activity on transient transfection; and (b) the C/EBP family of transcription factors and glucocorticoid receptor (GR) bind preferentially to this region of the promoter when nucleoside A is present at –217. In addition, variant –217A is always present with variants –532T, –793A, and –1074T in the human *AGT* gene promoter. We have also shown that liver enriched transcription factor HNF-3 $\beta$  binds more strongly when nucleoside T is present at –1074. Previous studies have shown that HNF-3 $\beta$  interacts with GR and plays an important role in liver-specific gene expression. These data suggest that *AGT* haplotype containing –217A, –532T, –793A, and –1074T may be involved in increased expression of this gene, and may play a role in human hypertension. It will be important to confirm this observation in future human studies and to understand the role of this haplotype in transcriptional regulation using transgenic animals.

**Key Words:** Human essential hypertension; molecular genetics; angiotensinogen gene; transcription factors; single nucleotide polymorphism (SNP); haplotype; C/EBP; HNF-3.

## INTRODUCTION

Hypertension is a serious risk factor for myocardial infarction, heart failure, vascular disease, stroke, and renal failure. It is estimated that hypertension affects 50 million Americans with a prevalence rate of 25–30% in the adult Caucasian population. In the United States, the prevalence of hypertension is 50% greater in blacks than in whites. In blacks, hypertension appears earlier, is generally more severe, and results in high rates of morbidity and mortality from stroke, heart failure, left ventricular hypertrophy, and end-stage renal disease. Both genetic and environmental factors may contribute to the higher prevalence of hypertension in blacks. In addition, a genetic contribution to the increased prevalence of renal disease among hypertensive blacks has been proposed on the basis of family studies and studies of associated histocompatibility antigens. Hypertension is a polygenic disease, and it has been estimated by segregation analysis and twin



studies that approx 45% of the interindividual differences in blood pressure (BP) can be accounted by genetic differences (1). In the past two decades, many genes that were implicated in simple (Mendelian) diseases have been identified using genetic linkage and positional cloning methods (2). Although these methods have been remarkably successful in identifying high relative risk genes, they have not been successful in identifying genes that are involved in the complex forms of disease such as hypertension and diabetes type II. This failure is the result of three main features of complex diseases. First, such diseases typically vary in severity of symptoms and age of onset, which results in difficulty in defining an appropriate phenotype and selecting the best population to study. Second, they can vary in the etiological mechanisms, which might involve various biological pathways. Third, and perhaps most importantly, complex diseases are more likely to be caused by several, and even numerous genes, each with a small overall contribution and relative risk. In addition, hypertension is an arbitrary definition and not a quantitative trait that appears relatively late in life. Nothing is known about the number of genes involved, their mode of transmission, their quantitative effect on BP, their interaction with other genes, or their modulation by environmental factors. Parameters such as ethnicity and body weight increase the genetic heterogeneity and the difficulty of replication from one study to another.

### GENOME-WIDE ANALYSIS TO IDENTIFY HYPERTENSION-RELATED GENES

Krushkal et al. (3) performed a genome-wide linkage analysis of systolic BP on 427 sibling pairs and identified four regions of the human genome that show statistical significant linkage. These regions are on chromosomes 2, 5, 6, and 15. These chromosomal regions include numerous potential candidates, such as phospholamban, estrogen receptor (ER), aminopeptidase *N*,  $\alpha$  1B-adrenergic receptor, dopamine receptor type 1A (DR-1A), calmodulin, and sodium-calcium exchanger. However, none of these genes has been confirmed to be associated with human hypertension.

Hunt et al. (4) performed a genome-wide analysis using 2959 individuals in 500 white families from the National Heart, Lung and Blood Institute (NHLBI) family heart study and identified five regions of the genome with logarithm of odds (LOD) scores higher than 2.0 for hypertension. These included chromosomes 1, 7, 12, and 15. On the other hand, chromosome 6 showed the best evidence for linkage with systolic BP (SBP). It is important to note that angiotensinogen and renin genes are located on chromosome 1.

Kristjansson et al. (5) performed a genome-wide scan with 904 microsatellite markers using 120 extended Icelandic families with 490 hypertensive patients. After adding 5 markers, they found linkage to chromosome 18q with an allele-sharing LOD score of 4.60. These results provide evidence for a novel susceptibility gene for essential hypertension on chromosome 18q, and show that it is possible to study the genetics of essential hypertension without stratifying by subphenotypes. Although melanocortin receptor 4 is located in this region of the chromosome, it is not known whether this gene is involved in hypertension.

Rice et al. (6) performed a genome-wide scan on 317 black individuals from 114 families and 519 white individuals from 99 families using a multipoint variance-components linkage model and a panel of 509 markers. Promising results were primarily, but not exclusively, found in the black families. Linkage evidence ( $p < 0.0023$ ) with baseline BP replicated other studies within a 1-LOD interval on 2p, 3p.3 and 12q.33.

Levy et al. (7) performed a genome-wide scan in the largest families from two generations of Framingham Heart study participants. Genotyping was performed on 1702 subjects from 332 large families, and BP data were available for 1585 (93%) genotyped subjects who contributed 12,588 longitudinal BP observations. For diastolic BP, LOD scores greater than 2.0 were identified on chromosome 17 (74cM, LOD 2.1) and chromosome 18 (7cM, LOD 2.1). Using a genome-wide scan, they found strong evidence for a BP quantitative trait locus on chromosome 17. The stroke-prone, spontaneously hypertensive rat and the Dahl salt-sensitive, hypertensive rat proved to be useful tools in identifying quantitative trait loci (QTL) that contribute to BP variations. Evidence of BP QTL was found on rat chromosomes 2, 10, and X. Human chromosome 17 is syntenic with rat chromosome 10, and the rat chromosome 10QTL is located near the angiotensin-converting enzyme (ACE) locus. The ACE locus is an attractive candidate gene because of its role in the renin-angiotensin system (RAS) and the association between several polymorphisms in the ACE locus with BP levels in some studies, although other studies failed to confirm this finding. Other potential candidate genes on this segment of chromosome 17 include the Cl-HCO exchanger, phenylethanolamine *N*-methyltransferase, nerve growth factor receptor, and pseudohypoaldosteronism type IIB genes.

Caulfield et al. (8) recently phenotyped 2010 affected sibling pairs drawn from 1599 severely hypertensive families in a large white European population (BRIGHT study). Linkage analysis identified a principle locus on chromosome 6q with an LOD score of 3.21, which attained genome-wide significance ( $p = 0.042$ ). The inclusion of three further loci with LOD scores higher than 1.57 (2q, 5q, and 9q) also show genome-wide significance ( $p = 0.017$ ) when assessed under a locus-counting analysis. There is a region of overlap with other studies on chromosome 2 (140–170 cM). This region was linked to hypertension in Chinese sibling pairs and Finnish twins, and linkage is suggested in a discordant sibling-pair screen. In addition, linkage signals are located proximally to the BRIGHT locus on chromosome 2p, documented by two genome scans in a genome scan of families of African ancestry. Furthermore, this region has also been linked to the hypertension-associated phenotype preeclampsia in some genome-wide screens. Preliminary analysis of each region with an LOD score higher than 1.57 has identified a small number of good candidates that map to the chromosome 2 and 9 regions of interest. These candidates include the genes encoding serine-threonine kinases (STK39, STK17B, chromosome 2q), a protein kinase (PKNBETA on 9q), G protein-coupled receptors (GPR21 on 9q33, GPR107 9q, and GPR1 on 2q33) and a potassium channel (KCNJ3 on 2q24.1). Several BP QTL from hypertensive rat models exhibit homology with the BRIGHT regions of interest. For instance, rat 3q11–q23 is homologous to the human 9q region, rat 2q14–q16 with the human chromosome 5p region, and rat chromosome 9q22–q34 is homologous to the human 2q locus. Examination of databases reveals many known genes and expressed sequence tags in or near the 16cM QTL interval at 17q12–21. Although no genes that have been strongly implicated in BP variation lie in this interval, potential candidate genes in the interval include the  $\alpha$ -1 thyroid receptors, the neuronal homolog of the amiloride sensitive epithelial sodium channel, the corticotropin-releasing hormone receptor 1, insulin-like growth factor-binding protein-4, hepatocyte nuclear factor (HNF) 1- $\beta$ , and the chloride/bicarbonate exchanger AE1. In addition to the chromosome 17q12–21 interval, there were two additional regions yielding LOD scores over 2.0 in multipoint analyses. One of these lies just distal on chromosome 17, and it is possible that this locus is independent of their largest peak. This second chromosome 17 peak overlaps the locus

encoding the ACE locus, a much-studied candidate gene for hypertension. The final locus that has not been previously implicated in BP variation is on chromosome 18. An interesting candidate gene in the chromosome 18 interval is the melanocortin receptor 2, which is the physiological receptor for corticotropin. Given the known effects of glucocorticoids on BP, it is of interest that receptors involved in the regulation of cortisol secretion lie in both the chromosome 17 and chromosome 18 intervals.

From this brief review it is clear that although different regions of the chromosome have been shown to be involved in the regulation of BP, these studies are often nonreproducible and genes involved in hypertension remain to be identified.

## ROLE OF AGT IN HYPERTENSION

The RAS plays an important role in the regulation of BP. The octapeptide, angiotensin II (Ang II), is one of the most active vasopressor agents and is obtained by the proteolytic cleavage of a larger precursor molecule, angiotensinogen (AGT), which is primarily synthesized in the liver and to a lesser extent in the kidney, brain, heart, adrenal, fat, and vascular walls. The human AGT cDNA is 1455 nucleotides long and codes for a 485-amino acid protein (9). AGT is first converted by renin to produce a decapeptide, Ang I, and is then converted to Ang II by the removal of a C-terminal dipeptide by ACE (10). In experimental as well as clinical studies, chronic administration of renin-angiotensin inhibitors has proven effective in lowering BP in hypertension. Genes that encode components of the RAS are therefore potential candidate genes that may play a role in the regulation of BP.

The plasma concentration of AGT is close to the Michaelis constant of the enzymatic reaction between renin and AGT (11). For this reason, a rise in plasma AGT levels can lead to a parallel increase in the formation of Ang II that may ultimately result in hypertension. In fact, recent studies have suggested a direct correlation between AGT and BP. These studies include a highly significant relationship between plasma concentration of AGT and BP in human subjects (4); higher plasma AGT levels in hypertensive subjects and in offspring of hypertensive parents compared with normotensives (12,13); expression of *AGT* gene in multiple tissues—such as brain, kidney, heart, adrenals, placenta, and vascular walls—that are directly involved in BP regulation (14); elevation of BP in transgenic animals that overexpress *AGT* gene (7–9); and reduction of BP in *AGT* gene knockout mice (15). In addition, Kim et al. have introduced up to four copies of the *AGT* gene in mice, with each copy of the gene resulting in a successive increase in BP (16). These results directly demonstrated that small increases in plasma AGT level can quantitatively influence the fine control of renal vascular resistance and increase BP in a gene dose-dependent manner. A similar *ACE* gene duplication in mice led to an increase in plasma ACE level but no increase in BP (17), which supports the importance of the *AGT* gene in human hypertension.

## M235T POLYMORPHISM OF AGT GENE AND HYPERTENSION

The *AGT* gene contains five exons and four introns, which span 13 kb (18). An extensive study of the potential role of the *AGT* gene in human essential hypertension was performed on two large series of hypertensive sibships yielding 379 sibling pairs. The highly polymorphic CA dinucleotide repeat marker located in the 3'-region of the *AGT* gene and the powerful affected sibling pair methodology were used to obtain evidence

of a genetic linkage between the *AGT* gene and hypertension in this study (19). A 17% excess of *AGT* allele sharing was found in severely hypertensive sibling pairs. Whereas significant linkage was obtained in male pairs in both the Utah and Paris groups, no excess of shared *AGT* alleles was observed in female subjects, suggesting the influence of an epistatic hormonal phenomenon. These studies also showed that variants 235T and 174M of the *AGT* gene are associated with hypertension. From these studies, it was estimated that mutations at the *AGT* locus might be a predisposing factor in at least 3–6% of hypertensive individuals younger than 60 yr of age in the Caucasian population. When the same methodology was used to analyze the same hypertensive sibling pairs from Utah or Paris, it showed no linkage with other genes of the renin system: namely renin (20), ACE (21), or Ang II type I receptor (22).

Two other linkage studies also indicate a relationship between the *AGT* locus and hypertension. This linkage was observed in the subgroup of patients with diastolic pressure above 100 mmHg, but there was no difference among female–female pairs. However, there was no association between hypertension and the 235T *AGT* variant in this population (23). The same group also found linkage and association of the *AGT* locus with high BP in 63 affected sibling pairs of African-Caribbean origin, suggesting some similarity in the genetic basis of essential hypertension in populations of different ethnicity (24). The corroboration of these linkage studies indicate that molecular variants of the *AGT* gene, such as M235T, or those in linkage disequilibrium with this variant are inherited predispositions to essential hypertension in humans.

However, results of these studies must be interpreted with caution for several reasons. First, the frequency of the 235T *AGT* allele varies in different ethnic groups. The 235T allele is more frequent in the Asian than in the Caucasian population and is by far the predominant allele in the African population. As a consequence, positive results may arise from population admixture, and negative results in populations in which this allele is predominant may result from the constraint of limited statistical power. Second, differences in the design of each study and the choice of the control group make any overall comparison difficult. Finally, most of the results reported to date have been obtained with relatively small numbers of patients, which can also generate false negative or false positive results.

There are divergent results on the association between variants in *AGT* at position 235 (235T) or 174 (174M) and hypertension in Caucasians. In their original study, Jeunemaitre et al. found that the M235T variant was more frequent in hypertensive probands from Utah and Paris, especially in the more severe index cases (19). A subsequent study by the same group on 136 mild to moderate hypertensive subjects also found that the frequency of the 235T allele was increased, although the increase was significant only for patients with a family history of hypertension. Schmidt also found a higher frequency of the 235T allele in subjects with hypertension, a family history of the disease, and early onset of hypertension (25).

However, other studies have not found any association between the 235T variant and hypertension. Hingorani et al. found no difference in the frequency of M235T in 223 hypertensive subjects and 187 normotensive individuals in Anglia in the United Kingdom (26). A more powerful study performed in Finland by Kiema gave negative results with 508 mild hypertensives and 523 population-based controls (27). On the other hand, four large studies recently showed a positive association between the M235T allele and essential hypertension. An association with severe hypertension with stronger relation-

ship for men than for women was found in a sample from the Framingham Heart study and from the Atherosclerosis Risk in Community (ARIC) study, when the effect of body mass index and triglycerides were taken into account (28). The proportions of cases attributable to the 235 allele were 8% in the ARIC population and 20% in the Framingham population. In a study testing a large number of AGT alleles, the frequency of the 235T allele was 0.47 in the 477 probands of hypertensive families and 0.38 in the 364 Caucasian controls (29). In a cross-section sample of 634 middle-aged subjects from the MONIC Angsburg cohort, Schunkert found that individuals carrying at least one copy of the T235 allele had high SBP and diastolic BP (DBP)s and were more likely to use antihypertensive drugs (30). Finally, another case-control study involving 802 hypertensive subjects and 658 Caucasian controls has shown a significant increase in the frequency of the T235 allele in men and in women whose hypertension was diagnosed before they were 45 yr of age (31). These studies emphasize the importance of sample size testing for susceptibility locus in a complex disease such as hypertension (32).

The frequency of the T235 allele is invariably high in the Japanese population, ranging from 0.65 to 0.75. An association between hypertension and molecular variants of AGT in the Japanese population has been found in several independent studies (33–35). All of the studies reported so far indicate that frequency of the 235T variant is higher in Japanese hypertensive subjects. A more homogeneous genetic and environmental background may explain why the results reported for Japanese populations are more uniform. However, Ishigami et al. (36) showed that the –20C variant of the human AGT gene plays an important role in hypertension in Japanese population.

Walker et al. showed a remarkably high correlation between plasma AGT concentration and elevated BP ( $p < 0.0001$ ) in a large study involving 574 black subjects (4). Bloem et al. (37) showed that (1) plasma AGT level is about 19% higher in black children as compared with white children; and (2) BP is normally higher and increases faster over time in black children as compared with white children. Caulfield et al. (24) found an association between the AGT gene locus and high BP in 63 affected sibling pairs of African-Caribbean origin using CA dinucleotide marker. However, these workers could not find an association between variants M235T and hypertension in the African-American population. Various recent studies have suggested that although the frequency of 235T allele is increased in the African-American population (0.8–0.9), there is no association between 235T allele and hypertension in this population (38).

### LINKAGE DISEQUILIBRIUM BETWEEN 235T AND –6A IN AGT GENE

Because amino acid 235 is located far away from the renin cleavage site, this polymorphism does not explain the molecular mechanism involved in increased plasma AGT levels by the 235T variant. The human AGT gene has an A/G polymorphism at nucleoside –6. It has been shown that nucleoside A is present at –6 in the ancestral AGT gene and –6G is a neomorph. Sequence analysis of the human AGT gene has shown that molecular variants 235T and –6A are in complete association (39). In addition, Inoue et al. synthesized reporter constructs containing 256 and 70 basepairs (bp) of the 5'-flanking region of the human AGT gene containing either nucleoside A or G at –6 position. Transient transfection of these reporter constructs in human liver cells (HepG2) have shown that reporter constructs containing human AGT gene promoter with nucleoside A at –6 have increased promoter activity compared to nucleoside G at –6. These experiments sug-

gested that increased plasma AGT levels by the 235T variant in hypertensive patients may actually be a result of increased transcriptional activity of the human *AGT* gene by nucleoside A at -6. However, the molecular mechanism involved in increased transcription of the human *AGT* gene by nucleoside A at -6 as compared with nucleoside G at -6 is not known, and transcription factors that may be involved in this process have not been identified. In addition, as discussed later, experiments using transgenic mice have shown that this polymorphism neither alters the expression of the *hAGT* gene nor increases BP in transgenic mice (40).

### ASSOCIATION OF THE -217A VARIANT OF *AGT* GENE WITH HYPERTENSION

Because hypertension is more common in African-American subjects, our laboratory is interested in understanding molecular mechanisms involved in hypertension in the African-American population. We have therefore analyzed 186 African-American and 127 Caucasian subjects with hypertension (mean age:  $59 \pm 10$  yr) and 156 African-American and 135 Caucasian normotensive controls (mean age  $58 \pm 10$  yr). All of these subjects were recruited from the outpatient department of The State University of New York Health Science Center in Brooklyn, New York and Westchester Medical Center, Valhalla, New York. All case and control subjects gave informed consent before participating in the research. All cases were diagnosed as having primary hypertension and patients with secondary hypertension, diabetes mellitus, or ischemic heart disease were excluded. The criteria for hypertension was defined as a SBP greater than 140 mmHg, a DBP greater than 90 mmHg, or under antihypertensive therapy. BP was measured twice with the subject seated and a 5-min interval between measurements. The normotensives (with SBP/DBP <140/90 mmHg) without a history of hypertension and without diabetes mellitus were recruited from the same population and matched for sex and age. All participants completed a standard questionnaire on personal medical history and family history of hypertension. All patients and control subjects were in Hardy-Weinberg equilibrium. The frequency of the -217A allele in hypertensive patients was 0.29 as compared with 0.19 in the normotensive population, which is highly significant ( $p = 0.0017$  and OR = 1.792) (Table 1). To compare the role of this polymorphic site on hypertension in the African-American and Caucasian populations, we also analyzed genomic DNA from 127 Caucasian hypertensive subjects and 135 normotensive controls. The frequency of -217A allele was 0.15 in Caucasian hypertensive subjects and 0.11 in normotensive controls which is not significant ( $p = 0.12$ ) (Table 1). Statistical analysis based on the -217 A/G genotype (using A allele as a dominant model) also suggested a significant role of the -217A allele in hypertension in African-Americans ( $p = 0.0021$  and OR = 2.015) and not in Caucasians (Table 2). Because an A/G polymorphism at -6 has previously been associated with hypertension, we also analyzed genomic DNA from the African-American and Caucasian populations for this polymorphism. The frequency of -6A allele was 0.87 in African-American hypertensive subjects and 0.85 in normotensive controls, which was not significant ( $p = 0.58$ ). However, the frequency of -6A allele was marginally significant in Caucasian subjects ( $p = 0.06$ ). These experiments suggested that -217A allele of the human *AGT* gene plays a significant role in essential hypertension in African-Americans (41). However, if we double the number of total Caucasian subjects, then the role of -217A becomes significant. It is therefore possible that this polymorphism will be significant in Caucasian subjects if we analyze more samples.

Table 1  
Statistical Analysis of -217 A/G Polymorphism of Human  
Angiotensinogen Based on Allele Frequency

	<i>A allele</i>	<i>G allele</i>	<i>p value</i>
African-American hypertensive ( <i>n</i> = 186)	0.29	0.71	<i>p</i> = 0.0017 OR = 1.792
Normotensive ( <i>n</i> = 156)	0.19	0.81	[1.247, 2.575]
Caucasian hypertensive ( <i>n</i> = 127)	0.15	0.85	<i>p</i> = 0.1208 OR = 1.507
Normotensive ( <i>n</i> = 135)	0.11	0.89	[0.901, 2.522]

Table 2  
Statistical Analysis of -217 A/G Polymorphism of Angiotensinogen Gene  
Based on the Genotype Distribution Using A Allele Dominant Model

	<i>(AA + AG)</i>		<i>GG</i>	<i>p value</i>
African-American hypertensive ( <i>n</i> = 186)	12	84	90	<i>p</i> = 0.0021 OR = 2.015
Normotensive ( <i>n</i> = 156)	4	50	102	[1.301, 3.121]
Caucasian hypertensive ( <i>n</i> = 127)	4	31	92	<i>p</i> = 0.1433 OR = 1.595
Normotensive ( <i>n</i> = 135)	3	23	109	[0.894, 2.844]

### TRANSCRIPTION FACTORS INVOLVED IN THE EXPRESSION OF *AGT* GENE

The activation of eukaryotic genes *in vivo* often requires the coordinated binding of multiple transcription factors to the promoter–enhancer region. It has been shown that distinct signal transduction pathways regulate activity of many of these transcription factors. In several cases, it has been shown that binding of multiple transcription factors to a specific promoter–enhancer region is cooperative and requires a unique composition and spatial arrangement of transcription factor binding sites. The assembly of these enhancer complexes is facilitated by protein–protein interactions between DNA-bound factors and protein-induced DNA bending. These features are important for transcriptional regulation because combination of multiple transcription factors generates a diverse pattern of regulation, and highly cooperative binding ensures the specificity of transcriptional control (42–44). Most eukaryotic transcription factors contain one or more transactivation domains that are involved in interaction with downstream coactivators (such as CREB binding protein, CBP/p300, and steroid receptor coactivators) and this interaction plays a crucial role in transcriptional activation of a gene (45,46). It is possible that polymorphisms in the promoter of a gene may affect the binding of transcription factors to this region of the promoter and alter the transcriptional regulation of the gene. For example, it has been shown that a single nucleotide polymorphism in the CD14

promoter decreases the affinity of SP1 transcription factor binding and enhances transcriptional activity (47). Similarly two mutations in the promoter of human protein-C gene cause type-1 protein-c deficiency by disruption of two HNF-3 binding sites (48). A polymorphism of the human matrix  $\gamma$ -carboxy glutamic acid protein promoter alters the binding of AP-1 complex and is associated with altered expression and serum levels of this protein (49).

The nucleotide sequence of the human *AGT* gene promoter containing nucleotide sequence from -1223 to +27 and potential *cis*-acting DNA elements that may bind to different transcription factors are shown in Fig. 1. Comparatively little is known about transcriptional regulation of the human *AGT* gene expression. We have shown that the nucleotide sequence located between -99 and -91 of the human *AGT* gene binds to the C/EBP family of transcription factors and this region of the promoter plays an important role in DBP and C/EBP- $\beta$ -induced expression of this gene (50). There is an adjacent SP1 binding site located around -87 and our recent data have suggested that SP1 and C/EBP- $\beta$  cooperatively regulate the expression of human *AG* gene. It has been previously shown that human *AGT* gene has a C/A polymorphic site at -20 (located between TATA box and transcriptional initiation site). We have shown that USF binds to this sequence when nucleoside C is present at -20 and ER binds to this sequence when nucleoside A is present at -20 (51). We have also shown that cotransfection of ER increases the expression of reporter constructs containing human *AGT* gene promoter with nucleoside A at -20 as compared with the same reporter constructs containing nucleoside G at this position. Orphan receptor Arp-1 also binds to this sequence and reduces ER-induced promoter activity (52). Yanai et al. have also shown that the nucleotide sequence located between the TATA box and transcriptional initiation site of the human *AGT* gene binds to USF and plays a critical role in its expression (53). In addition, we have shown that the liver-enriched transcription factor HNF-3 binds to the nucleotide sequence located between +10 and +20 of the human *AGT* gene promoter (54). It has been shown recently that human *AGT* gene expression is increased by interleukin (IL)-6 treatment and a STAT-3 binding site is located at -274 (55). Our gel shift and transient transfection assays have shown that nucleotide sequence around -240 is an HNF-1 site. HNF-1 plays an important role in liver and kidney specific expression of a gene. Nucleotide sequence of the human *AGT* gene around -217 contains a full palindromic GRE and there are three glucocorticoid receptor-binding site (GRE), half sites in other regions of the promoter (at -673, -130 and +15). We have also shown that the transcription factor CREB binds to the nucleotide sequence located between -840 and -830 of the human *AGT* gene and this sequence is involved in cAMP-induced expression of this gene (56). This is a composite element and has sequence homology with a C/EBP binding site. Nucleotide sequence located between -323 and 490 contains multiple hormone receptor binding sites. There are at least three HNF-4 binding sites (DR-1) between -363 and -423. In addition, this sequence contains DR-4 sites that may be involved in binding with thyroid hormone receptor. Although it has been shown that this region of the promoter is important in the regulation of *AGT* gene expression by HNF-4 (57), it is not known whether it plays any role in TR-induced expression of this gene. It has been shown previously that CREB, HNF-4, ER, USF, C/EBP and GR (which bind to different regions of the human *AGT* gene) interact with transcriptional coactivator CBP and coordinately regulate the expression of a gene. However, the role of CBP in transcriptional activation of human *AGT* gene, which is regulated by multiple transcription factors that can individually bind to CBP, is not known.



*AGT* gene is expressed in multiple tissues such as liver, fat, kidney, brain, and adrenals. However, molecular mechanisms involved in tissue specific expression of this gene are not known. It has been shown that the rat *AGT* gene expression is regulated by glucocorticoids, estrogens, androgens, and thyroid hormones, and by different cytokines during inflammation (10). Glucocorticoids are the most important regulatory agents, and cell culture studies have shown that they increase the expression of *AGT* gene in liver and fat cells. Glucocorticoids can modulate the expression of *AGT* gene by interaction with multiple transcription factors. It has been shown that GR interacts with STAT-3 and C/EBP- $\beta$ . Because IL-6 increases the expression of a gene through STAT-3 and C/EBP pathways, glucocorticoids may increase the expression of *AGT* gene through the action of IL-6. In addition, GR interacts with cAMP and increases the expression of various liver specific genes. In the case of rat *AGT* gene, a nucleotide sequence called acute phase responsive element (APRE) is located around -545 (58). This nucleotide sequence is flanked by two GREs: a full palindromic sequence located around -580, and a half palindromic sequence located around -470. The nucleotide sequence of APRE has strong homology with NF $\kappa$ B site and partial homology with C/EBP binding site. Gel shift analysis has shown that both of these transcription factors can bind to APRE. Under basal conditions, a constitutively synthesized transcription factor (most probably C/EBP- $\alpha$ ) binds to this sequence. However, on lipopolysaccharide (LPS) treatment, an inducible factor (most probably NF $\kappa$ B) binds to this sequence and displaces C/EBP- $\alpha$ . Transient transfection analysis has shown that presence of both of the GREs is required for maximum increase in the expression of rat *AGT* gene by IL-1 and LPS treatment. These experiments suggested that expression of the rat *AG* gene is increased during acute phase reaction mainly through the NF $\kappa$ B pathway. On the other hand, Brasier's group has shown that expression of the human *AG* gene is increased mainly by the IL-6 pathway (55). However, the molecular mechanism involved in the regulation of human *AGT* gene expression by glucocorticoids is not known, and the role of GREs in this gene has not been analyzed.

The promoters of rat and human *AGT* genes are very different with respect to the location and sequence of GRE. The palindromic GRE of the rat gene has stronger homology with the consensus GRE, but is located far away from the transcriptional initiation site, at -580. On the other hand, although the GRE in the promoter of human *AGT* gene has only partial homology with consensus GRE, it is located much closer to the transcriptional initiation site, at -217. This site in the human gene is located in close proximity with the C/EBP and STAT-3 binding sites.

### HUMAN *AGT* GENE PROMOTER WITH -217A VARIANT HAS INCREASED PROMOTER ACTIVITY

To understand the role of A/G polymorphism at -217 on transcription of the human *AGT* gene, we have synthesized reporter constructs where different regions of the human *AGT* gene promoter were attached to the luciferase gene in pGL3 basic vector (Promega Biotec). This vector does not contain any promoter or enhancer sequence. The reporter construct pHAGT1.3*luc* has 1223 bp of the 5'-flanking sequence and 70 bp of exon-I of the human *AGT* gene, and contains nucleoside A at -6 and G at -217. This construct was then mutated from -217G to -217A using a STRATAGENE site-specific mutagenesis kit. The resulting reporter construct had nucleoside A at -6 and at -217 as determined by nucleotide sequence analysis. These reporter constructs were transiently transfected in

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-1223    CCAGACAAGT GATTTTTGAG GAGTCCTAT CTATAGGAAC AAAGTAATTA
-1173    AAAAAATGTA TTTCAGAATT ATACAGGCCA TGTGAGATAT GATTTTTTTA
                                     HNF-3
-1123    AATGAAGATT TAGATAATG GGTAAAAAAG AGGTATTTGT GTGTTTGTG*
-1073    ATTGTTCAAGT CAGTGAATGT ACAGCTTCTG CCTCATATCC AGGCACCATC
-973     TGCCATCGTG GATATGCCGT GGCTCCTTGA ACCTGCTTGT GTTGAAGCAG
-923     GATCTTCTT CCTGTCCCTT CAGTGCCCTA ATACCATGTA TTTAAGGCTG
                                     AP-1          CREB
-873     GACACATCAC CACTCCCAAC CTGCCTCACC CACTGCGTCA CTTGTGATCA
-823     CTGGCTTCTG GCGACGTCTCA CCAAGGTCTC TGTGATGCCC TGTATAACG*
-773     ACTACAAAG CAAGTCTTAC CTATAGGAAA ATAAGAATTA TAACCCTTTT
-723     ACTGGTCATG TGAAACTTAC CATTGGAAT TTGTACAGCA TAAACACAGA
-673     ACAGCACATA TTTCAATGCC TGCATCCTGA AGGCATTTTG TTTGTGTCTT
-623     TCAATCTGGA TGTGCTATTG TTGGTGTTTA ACAGTCTCCC CAGCTACACT
-573     GGAAACTTCC AGAAGGCACT TTCACTTGC TTGTGTGTTT TCCCCAGTG*
-523     CTATTAGAGG CCTTTGCACA GGGTAGGCTC TTTGGAGCAG CTGAAGGTCA
-473     CACATCCCAT GAGTGGGCAG CAGGGTCAGA AGTGGCCCCC GTGTTGCCTA
                                     HNF-4/PPAR
-423     AGCAAGACTC TCCCCTGCCC TCTGCCCTCT GCACCTCCGG CCTGCATGTC
                                     SP1/AP-1
-373     CCTGTGGCCT CTTGGGGGTA CATCTCCCGG GGCTGGGTCA GAAGGCCTGG
                                     APRE
-323     GTGGTTGGCC TCAGGCTGTC ACACACCTAG GGAGATGCTC CCGTTTCTGG
                                     HNF-1
-273     GAACCTTTGGC CCCGACTCCT GCAAACCTCG GTAAATGTGT AACTCGACCC
                                     CEBP/GRE
-223     TGCACCGGCT CACCTCTGTT AGCAGTGAAA CTCTGCATCG ATCACTAAGA
                                     APRE *          GRE
-173     CTTCTGGGAA GAGGTCCCAG CGTGAGTGTC GCTTCTGGCA TCTGTCCTC
                                     CEBP
-123     TGGCCAGCCT GTGGT CTGGC CAAGTGATGT AACCCCTCCTC TCCAGCCTGT
-73     GCACAGGCAG CCTGGGAACA GCTCCATCCC CACCCCTCAG CTATAAATAG
                                     ERE/USF          +1
-23     GGCATCGTGA CCCGGCCGGG GGAAGAAGCT GCCGTTGTTC TGGGTACTAC
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**Fig. 1.** Nucleotide sequence of the 1223 bp 5'-flanking region and 27 bp of the first exon of human angiotensinogen gene. Potential *cis*-acting DNA elements that may be involved in transcriptional regulation of this gene are underlined. The transcriptional initiation site is marked as +1.

HepG2 cells. The promoter activity was analyzed after 48 h of transfection, and normalized with the  $\beta$ -gal activity. Results of this experiment showed that reporter construct pHAGT1.3*luc* with nucleoside A at -217 gave a 28% increase in the promoter activity

as compared with the reporter construct pHAGT1.3*luc* with nucleoside G at -217 ( $p < 0.001$ ) (mean value of six experiments) (41). We have recently used these reporter constructs in transient transfection in Hep3B (another human liver-derived cell line) and immortalized human hepatocytes (TPH cells obtained from Dr. Ranjit Ray). TPH cells were obtained by transduction of Hepatitis C virus core protein in primary human hepatocytes, and we have shown that these cells synthesize human AGT mRNA by RT-PCR. Results of these experiments have shown that the reporter construct containing -217A had 20–30% increased basal promoter activity as compared with the reporter construct containing -217G.

### NUCLEOTIDE SEQUENCE OF *AGT* GENE AROUND -217 HAS HOMOLGY WITH GRE AND C/EBP BINDING SITE

The nucleotide sequence of human *AGT* gene promoter from -203 to -226 containing A/G polymorphic site at -217 is shown in the top line of Fig. 2. It has homology with the C/EBP binding site (the consensus C/EBP binding site TT/GNNGCAAT/G is shown in the reverse orientation in line 2). The -217 polymorphic site is the first nucleotide of the consensus C/EBP site. In addition, a consensus GRE (third line) is present in this region of the promoter. The first nucleoside of the palindromic GRE corresponds to the polymorphic site -217 of the *AGT* gene promoter. It has been shown previously that GR cooperatively interacts with other transcription factors and this combinatorial interaction is involved in glucocorticoid induced promoter activity in PEPCK and TAT genes. It has also been shown that C/EBP- $\beta$  and CREB can interact with GR and cooperatively increase the GR-induced promoter activity by cAMP.

In order to examine whether polymorphism at -217 affects the binding of transcription factors to this region of the promoter, we synthesized two oligonucleotides containing either nucleoside A or G at -217 position and used them in gel shift assays. Our gel shift analysis has suggested that the oligonucleotide containing nucleoside A at -217 binds more strongly to C/EBP family of transcription factors and GR as compared to the same oligonucleotide containing nucleoside G at this position (41).

### GLUCOCORTICOIDS AND C/EBP INCREASE THE PROMOTER ACTIVITY OF -217A VARIANT

In order to understand the physiological significance of the GR binding site at -217 region of the *hAGT* gene promoter, we synthesized reporter constructs where six copies each of either oligonucleotide 223A or 223G were attached in front of the pGL2-*luc* vector (obtained from Promega). These reporter constructs were transiently transfected in HepG2 cells in the presence of an expression vector that contained rat GR coding sequence. After transfection, cells were treated with 100 nM dexamethasone, and promoter activity was analyzed after 24 h. Results of this experiment showed that promoter activity of reporter construct containing -217A increased by about 18-fold over the basal value. On the other hand, promoter activity of reporter construct containing -217G increased only two- to threefold. Because PGC-1 acts as a coactivator and increases GR induced promoter activity, we also cotransfected an expression vector containing hPGC-1 in this experiment.

```

-226           -217           -195
C C C T G C A C C A / G G C T C A C T C T G T T C A G C A G T G A A   AGT Gene
                               Cons C/EBP
C T T G C N N C A
                               Cons GRE
A G A A C A N N N T G T T C T

```

Nucleotide sequence homology between human *AG* gene promoter around nucleotide polymorphism A/G at -217 with consensus C/EBP and GR binding sites

**Fig. 2.** Nucleotide sequence homology between human *AG* gene promoter around nucleotide polymorphism A/G at -217 with consensus C/EBP and GR-binding sites.

Results of this experiment showed that PGC-1 further increased the GR induced promoter activity. The same results were obtained by transient transfection in other cells such as human embryonic kidney cells (HEK 293) and 3T3L1. Taken together, results of these experiments suggested that reporter a construct containing six copies of an oligonucleotide containing -217A has increased GR induced promoter activity as compared with the reporter construct containing six copies of an oligonucleotide containing 217G.

Because C/EBP- $\beta$  and - $\delta$  play an important role in acute phase reaction, and *AGT* gene expression is upregulated during acute phase reaction, we were interested in studying the effect of these transcription factors in the expression of human *AGT* gene containing A/G polymorphic site at -217. Expression vectors containing the coding sequence of either C/EBP- $\beta$  or C/EBP- $\delta$  were cotransfected with reporter constructs containing different regions of the human *AGT* gene linked to the luciferase gene containing either nucleoside A or G at -217 in HepG2 cells. Transfected cells were then treated with IL-6 and promoter activity was analyzed by luciferase assay. Results of cotransfection experiments with C/EBP- $\beta$  show that reporter construct pHAGT1.3*luc* containing nucleoside A at -217 had 30% increased promoter activity and reporter construct pHAGT303*luc* containing nucleoside A at -217 had 43% increased promoter activity as compared with corresponding reporter constructs containing nucleoside G at -217. Similarly, results of co-transfection experiments with C/EBP- $\delta$  show that reporter construct pHAGT1.3*luc* containing nucleoside A at -217 has 17% increased promoter activity, reporter construct pHAGT303*luc* containing nucleoside A at -217 had 32% increased promoter activity as compared with corresponding reporter constructs containing nucleoside G at -217 (41). It is possible that combinatorial interaction of C/EBP, GRE, and STAT-3 is involved in the induced expression of the human *AG* gene by the IL-6 pathway, and polymorphism at -217 may play an important role in this process.

### ASSOCIATION OF VARIANT -217A WITH OTHER POLYMORPHIC SITES OF *AGT* GENE

Previous studies have identified seven polymorphic sites in the 1.2 Kb promoter of human *AGT* gene (29). These polymorphic sites are A/G at -6, A/C at -20, C/T at -532, C/T at -776, G/A at -793, T/A at -830, and G/T at -1074, and are shown in Fig. 3. Jeunemaitra et al. (29) have shown that polymorphisms at -532, -793, and -1074 always occur together. Surprisingly, these authors did not find the A/G polymorphism at -217 in the *AGT* gene promoter. In order to understand the linkage disequilibrium of these polymorphic sites in African-American subjects, we have performed sequence analysis of DNA samples isolated from African-American subjects and have found that variant



**Fig. 3.** Position of single nucleotide polymorphisms in the 1.2 kb promoter region of the human angiotensinogen gene.

-217A always occurs with -532T, -793A, and -1074G. The frequency of haplotype AA was 0.237 and that of haplotype AG was 0.086 in African-American subjects, which was statistically significant. This suggests that transcription factors binding to these sites in the *AGT* gene promoter may interact with each other and regulate the expression of this gene in subjects that contain allele -217A.

### NUCLEOTIDE SEQUENCE AROUND -1074T OF *AGT* GENE HAS HOMOLGY WITH HNF-3 BINDING SITE

The nucleotide sequence of *AGT* gene promoter around -1074 has partial sequence homology with the consensus HNF-3 binding site (Fig. 4). The nucleotide sequence of *AGT* gene with nucleoside G at -1074 has two mismatches with the consensus HNF-3 binding site, whereas the nucleotide sequence of *AGT* gene with nucleoside T at -1074 has one mismatch. Liver-enriched transcription factor HNF-3 belongs to the winged helix/forkhead family of transcription factors, which plays an important role in transcription of liver specific genes. Previous studies have suggested that HNF-3 acts as an accessory factor in GR-induced promoter activity of phosphoenolpyruvate carboxykinase (*PEPCK*) and *TAT* genes in the liver cells. Our unpublished data has shown that an oligonucleotide from this region of the promoter binds more strongly to HNF-3 $\beta$  when nucleoside T is present at -1074. It is tempting to speculate that increased binding of GR and C/EBP $\beta$  at -217A and increased binding of HNF-3 $\beta$  at -1074T is involved in increased expression of the *hAGT* gene in this haplotype.

### HAPLOTYPE -6A: -217A: -1074 OF *AGT* GENE HAS INCREASED PROMOTER ACTIVITY

Because variant -217A always occurs in association with -532T, -793A, and -1074T, we were interested in examining whether *AGT* gene promoter containing this haplotype AA (containing nucleoside A at -6 and A at -217) has increased promoter activity as compared with the haplotype AG (containing nucleoside A at -6 and nucleoside G at -217). For this purpose, we amplified 1.3 Kb *AGT* gene promoter from a hypertensive and a normotensive subject. These amplified sequences contained -6A, -20A, -217A, -532T, -776T, -793A, and -1074T (haplotype AA) and -6A, -20A, -217G, -532C, -776T, -793G, and -1074G (haplotype AG). These amplified sequences were ligated in pGL3*luc* to produce reporter constructs containing these haplotypes. These reporter constructs were then used in transient transfections in HepG2, Hep3B, TPH, and differentiated and undifferentiated 3T3L1 cells. In all the cells, haplotype AA gave increased basal promoter activity as compared with haplotype AG. Because immortalized human liver cells are the closest representatives of the primary human liver cells, results of this experiment are

**Homology between nucleotide sequence around T/G polymorphic site  
at -1074 of human AGT gene promoter with HNF-3 binding site**

T A T T T/G A/G T/C T T/C      CONS. HNF-3 SITE  
T G T T T    G    T T T/G      AGT GENE

**Fig. 4.** Homology between nucleotide sequence around T/G polymorphic site at -1074 of human AGT gene promoter with HNF-3 binding site

presented here. Reporter constructs (1  $\mu$ g) and RSV-gal (50 ng) were cotransfected in TPH cells (400,000 cells per well) in triplicates using six-well plates in the presence of lipofectamine reagent (Qiagen). The promoter activity was analyzed after 48 h of transfection and normalized with the  $\beta$ -gal activity. Results of this experiment showed that the promoter activity of haplotype AA was 1.9-fold higher than that of haplotype AG.

**TRANSGENIC MICE TO STUDY THE EFFECT  
OF POLYMORPHISMS OF AGT GENE IN VIVO**

Transient transfection in cultured cells has provided important information about tissue-, hormonal-, and developmental-specific transcriptional regulation of a number of genes. However, such studies are often hampered by the limited selection and state of differentiation of cell lines used in transient transfection assay. For example, transcription rates of numerous liver-specific genes are greatly reduced in cultured hepatoma cells and primary hepatocytes. In many instances, cultured cells have lost functional hormone receptors (such as glucocorticoid, estrogen, and angiotensin-II receptors in HepG2 cells) and expression vectors containing coding region of these receptors have to be cotransfected to analyze the effect of a particular hormone. These experiments raise another serious problem—the number of transfected receptors may far exceed its physiological level in transfected cells, and may provide false information. Transgenic mice are at present the most rigorous system available for identifying and characterizing *cis*-acting DNA elements. In addition, transgene expression can be assessed in numerous cell types as well as during both embryonic and fetal development.

The transgenic-mice approach has been successfully used to identify *cis*-acting DNA elements involved in liver, kidney, and adipose-specific expression of the rat *PEPCK* gene (59,60). These studies have also provided information about *cis*-acting DNA elements involved in developmental, glucocorticoid, and dietary-regulated expression of this gene. These transgenic experiments have complemented some of the results obtained by transient transfection assay in an *in vivo* setting. In some cases, mutations were made in selected *cis*-acting DNA elements and the effect of these mutations on tissue-specific expression of the mutated promoter was analyzed by the expression of transgene in mice (60). In recent years, transgenic mice have been generated by the injection of fusion genes containing promoter sequences attached to the  $\beta$ -galactosidase gene. These transgenic mice provide a better colorimetric assay to analyze the expression of transgene in different tissues. A fusion gene was synthesized by attaching the *lac Z* gene in front of a thymidine kinase promoter sequence attached to a NF-IL6 binding site. Transgenic mice containing this fusion gene were then used to study the effect of hypoxia in an *in vivo*

situation. Results of these experiments indicated that expression of the fusion gene containing the NF-IL6 sequence was increased under hypoxic conditions as measured by the  $\beta$ -gal assay. On the other hand, transgenic mice containing a mutated NF-IL6 site did not show an increase in  $\beta$ -gal activity under hypoxic conditions (61). In a similar way, cell-specific expression of the mouse glycoprotein hormone  $\beta$ -subunit gene was analyzed by *lac Z* gene expression of expression vectors containing a different region of its promoter in transgenic mice (62).

One of the main problems in these transgenic studies is that transgene can integrate at different sites in the chromosome and, depending on the site of integration, promoter activity may vary from experiment to experiment. Another problem is that a different number of transgene copies may integrate in the genome of different mice and promoter activity may vary depending on the number of transgenes in a particular line. However, experiments with *PEPCK* gene have shown that promoter activity did not vary significantly as a result of the number of transgenes in different animals.

In order to overcome these limitations, Sigmund's group has developed another experimental model system to assess the physiological significance of the human *AGT* gene (63). In this model, gene targeting at the *HPRT* locus was used to selectively target a single copy of the human *AGT* gene to a known site in the genome. They showed that insertion of the single copy transgene upstream of *HPRT* does not affect the overall tissue- and cell-specific expression or hormonal regulation of human *AGT*. This study provides an important proof-of-principle that the functional significance of allelic variation in human *AGT* can be assessed by examining mice carrying transgenes targeted in a single copy to an identical insertion site. They targeted the human *AGT* gene to the mouse *HPRT* locus by employing embryonic stem (ES) cells harboring a deletion in the endogenous *HPRT* gene, and a special targeting vector capable of restoring its full functionality upon homologous recombination. The use of gene targeting is essential in developing a model for studying effects of allelic gene variants *in vivo* because it nullifies the copy number and positional effects associated with transgene expression in transgenic mouse models generated by pronuclear injection. It allows reproducible insertion of the single copy of a transgene in a predetermined locus, permitting direct comparison with individually generated transgenic lines. Furthermore, independently derived transgenic mice generated by gene targeting are genetically identical, abolishing the need for multiple transgenic mouse lines harboring the same construct. An important practical benefit of their strategy is the highly reproducible efficiency in selecting single homologous recombination events. This is because, unlike the common approach to homologous recombination in which the targeting construct carries a positive selectable marker, the *HPRT* system carries sequences that can repair the deletion of *HPRT* found in the specific ES clone that is suitable for these studies. The selection for growth in HAT medium allows only targeted clones to survive, so all clones that survive selection have been targeted. This should allow for relatively easy development of a number of transgenic ES cell clones that harbor specific variants of a gene of interest, and the subsequent generation of transgenic mice.

However, there are certain potential drawbacks of using a nonnative locus, such as *HPRT*, for gene targeting. Exogenous genes may not harbor all of the sequences necessary and sufficient for proper regulation of expression and may therefore be influenced by *cis*-acting regulatory elements in the vicinity of the *HPRT* gene. However, previous studies by Sigmund's group have shown that the 13.8-kb genomic human *AGT* transgene

is expressed in a tissue- and cell-specific and copy-number proportional fashion in transgenic mice, suggesting that all essential regulatory elements are present in the transgene. Another potential drawback of targeting the HPRT locus is that it is located on the X chromosome and the transgene expression is affected by random X inactivation. This means that one can effectively achieve an equivalent of 1°copy (in the case of male and homozygous transgenic female mice) or 0.5°copy (in the case of heterozygous transgenic female mice) transgene expression. Taken together, their data indicated that the human *AGT* gene was apparently unaffected by the HPRT locus. It exhibited correct tissue and cell specificity and was properly regulated by the changes in testosterone levels. Furthermore, the human *AGT* protein was normally processed, and its plasma concentration was proportional to the level of human *AGT* mRNA, indicating proper regulation. The protein in plasma is readily cleaved by human renin, resulting in the acute pressor response, which could be easily detected by changes in BP.

In a recent paper, this group has used this model to specifically test the physiological effects of variants at nucleoside -6 and amino acid 235 in the *AGT* gene in vivo (40). For this purpose, they generated two different h*AGT* transgenes, representative of the two haplotypes observed in humans, by site-directed mutagenesis, and inserted both transgenes in a single copy at HPRT in the mouse genome. The identical genetic environment in both genetic background and transgene location allowed them to directly compare the transcriptional and functional activity of each haplotype and to link any significant difference in phenotype(s) to the sequence differences of the h*AGT* transgenes. The molecular analysis of the two h*AGT* haplotypes showed that variation at nucleotide -6 and amino acid 235 positions have no effect on the tissue- and cell-specific expression pattern in either males or females, and that both variants are transcribed with equal efficiency in liver, kidney, brain, and aorta. This result was unexpected, because previous reports indicated that the -6 variant affects baseline transcriptional efficiency both in vitro (in the context of a reporter construct), and in vitro, in that it may influence the steady-state level of *AGT* mRNA in the decidual spiral arteries of first trimester pregnant women. They have argued that there are several possibilities for this disparity. As was previously shown by the work in their laboratory, the h*AGT* gene can behave differently when studied in vitro and in vivo (46). Whereas deletion mutations in the promoter and 3' enhancer of the h*AGT* gene were shown to have an obvious effect on the transcription of the gene in HepG2 cells, no effect on gene regulation was detected in vivo when these mutations were studied in transgenic mice. The reason for this remains elusive, although it is conceivable that the HepG2 cell culture model is lacking all of the factors involved in the normal regulation of the h*AGT* expression, because it is an immortalized cell line. It is also possible that the environment of a mouse cell does not allow for the effect of the h*AGT* variants to be revealed. This could result either from the absence of cellular factors that can recognize specific elements of the h*AGT* promoter, or the fact that those factors are significantly different from their human counterparts so that they cannot serve the same function.

It is important to recognize that Sigmund's group has specifically analyzed the effect of only one polymorphism, namely at -6 position, in the h*AGT* gene on its transcriptional regulation (40). However, studies by Inoue et al. have shown the effect of only 256 bp of the 5'-flanking region of the human *AGT* gene on transient transfection in HepG2 cells. It is important to note that the effect of -6A variant was very small (only 14–19%) on transient transfection. It is possible that experiments using transgenic animals containing different haplotypes of the human *AGT* gene may provide meaningful results. It is also



important to mention that in order to examine the effect of promoter variants of the *AGT* gene on the BP in transgenic animals, one has to use double transgenic mice containing human *AGT*, as well as human renin genes. Very little is known about the tissue-specific regulation of human renin gene expression and therefore, although these experiments are very interesting, there are many unsolved problems. In addition, mouse physiology may be different from the human physiology and this haplotype, though important in the regulation of BP in human subjects, may not show its effect in mice.

## CONCLUSIONS AND PERSPECTIVES

Hypertension is a polygenic disease, and most probably multiple genes are involved in the etiology of this disease in different ethnic groups. Although initial studies, based on microsatellite markers, had shown that renin, ACE, and angiotensin receptor type 1 genes are not involved in hypertension, recent studies have suggested that certain polymorphic variants of renin and angiotensin receptor may be involved in human essential hypertension (64). It is also important to mention that other genes such as adducin (65),  $\beta_2$ -adrenergic receptor (66), and G protein  $\beta_3$  subunit (67) have also been suggested to be involved in human hypertension. It will be important to examine whether interaction of these genes leads to hypertension.

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## Substitution Mapping

*Using Congenic Strains to Detect  
Genes Controlling Blood Pressure*

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*Bina Joe, PhD*

*and Michael R. Garrett, MS, MBA*

### CONTENTS

INTRODUCTION

RESULTS

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### SUMMARY

Changes in blood pressure (BP) from low to high, are genetically controlled by many genes. Identifying genes that regulate BP is important for two major reasons: (1) for delineating the biochemical/physiological mechanisms for BP control; and (2) for utilizing this knowledge to improve clinical management strategies for maintenance of normal physiological BP, which is an important factor in reducing the risk for susceptibility to cardiovascular diseases.

Genetic approaches to identify BP regulatory genes involve the use of rat models with elevated BP (hypertension). Typically, genetic linkage analyses and substitution mapping using congenic strains are performed. These studies are designed to enable the identification of BP regulatory genes primarily by virtue of their location on the rat genome. This chapter captures the current status of studies aimed at identifying BP causative genes using various congenic rat strains.

**Key Words:** Hypertension; genetic; rat; animal model; QTL; cardiovascular; renal; disease causative genes.

### INTRODUCTION

#### *Monogenic vs Polygenic Diseases*

Depending on the number of genes that are responsible for causing disturbances in normal physiology, diseases can be classified as being (1) monogenic, i.e., caused by a

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single gene; or (2) polygenic, i.e., caused by many genes. An important aspect of molecular genetics involves the identification of genes causing disease irrespective of whether they are monogenic or polygenic. The first step in discovering disease causative genes involves mapping the gene precisely to a small chromosomal interval. Mapping genes causing monogenic diseases is easier than mapping genes causing polygenic diseases because of the strong correlation between phenotype and genotype. Single recombinants are sufficient to define minimal chromosomal intervals (where the causative gene resides) to less than 1 centimorgan (cM). As a result, discovery of DNA sequence variants that are found only in one of a small number of candidate genes in affected subjects usually provides adequate evidence to establish identity of the disease-causing gene. This technique has been successfully used for gene identification of many monogenic diseases in humans. These are compiled at the following website: <http://www.ncbi.nlm.nih.gov/books/bv.fcgi?call=bv.View..ShowSection&rid=gnd>; additional information is also available at <http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=OMIM>.

Discovering genes that cause polygenic diseases involves basically the same strategy as for monogenic disorders, but it is not as straightforward (1). When many genes control a single trait, the phenotype–genotype correlations at a gene locus are not nearly as strong as they are for monogenic diseases. This is because a single locus does not define the polygenic trait, but rather it is the net effect over multiple loci, which defines the trait. Moreover, finding genes causing polygenic diseases in humans is further confounded by a variety of factors such as gene–gene interactions, gene–environment interactions, genetic heterogeneity among populations, and incomplete disease penetrance. Considering these complexities combined with the difficulty associated with finding causative genes for polygenic traits, it is a generally well-accepted norm to add the word “complex” as a prefix when referring to polygenic traits/diseases. To date, only a handful of causative genes for complex polygenic diseases have been identified (2,3).

### ***Using Rat Models for Understanding the Genetic Basis of Blood Pressure Regulation***

Blood pressure (BP) in humans and rodents is well-recognized as a “quantitative trait” (i.e., variable from low to high) under polygenic control (i.e., controlled by many genes) (4). High BP (hypertension) is an undesirable trait because it is a major risk factor for cardiovascular and renal diseases. The expected result from finding and characterizing the genes that control BP is the gaining of insights into the etiology of hypertension. Several of the complications (such as those listed in the previous paragraph) that are associated with genetic studies of BP in humans are reduced to a large extent in well-defined experimental animal models of hypertension, which therefore serve as useful alternates for the genetic analysis of BP in humans.

Inbred strains of hypertensive rats are used as genetic models to identify genes that control BP. These include the Dahl salt-sensitive (S) rat, the spontaneously hypertensive rat (SHR), stroke-prone SHR (SHRSP), Milan hypertensive strain (MHS), genetically hypertensive (GH) rat, Sabra DOCA salt-sensitive (SBH) rat, Lyon hypertensive (LH) rat, Fawn-hooded hypertensive (FHH) rat, inherited stress-induced arterial hypertension (ISIAH) rat, albino surgery (AS) rat, and the Prague hypertensive rat (PHR) (5). Each of these rat strains represents an individual genetic pool of naturally occurring alleles that account for an overall phenotype of high BP. Determining the identity of each of these BP-increasing alleles is important in order to understand the molecular underpinnings of

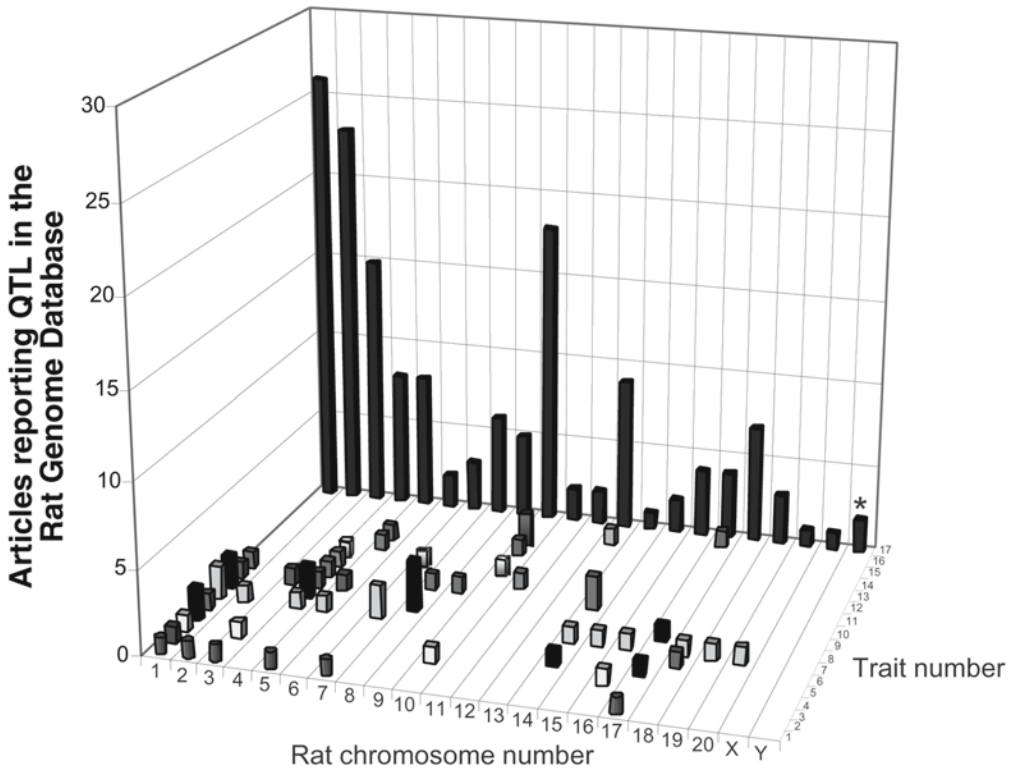
BP regulation. Genetic experiments to do so involve mapping the location of the BP causative genes on the rat genome with progressively improving resolution. These experiments are detailed in the following stages.

### STAGE 1: GENETIC LINKAGE ANALYSIS

The first stage is to establish statistically significant genome-wide evidence for linkage of large regions of the genome to BP. Genomic regions identified to contain genes that regulate a quantitative trait such as BP are called quantitative trait loci (QTL). BP QTL can be identified by whole-genome genetic linkage analysis in segregated populations of hypertensive and relatively normotensive rats. Using this approach, information on chromosomal location, the magnitude of the BP effect, and the mode of inheritance of each causative locus can be obtained. It was, however, not until 1991 that the first full genome scans for BP QTL were done by Hilbert et al. (6) and Jacob et al. (7) using microsatellite markers on the same F2 population derived from hypertensive SHRSP rats and Wistar Kyoto (WKY) rats. Subsequently, multiple genetic linkage analyses have been performed using various hypertensive-inbred strains. Results from these studies indicate that there are BP QTL widely distributed throughout the rat genome, as is summarized at the following website: <http://rgd.mcw.edu/qtls/>. Figure 1 summarizes the numbers of articles on QTL that control BP and related phenotypes, sorted by their location on the rat genome by chromosome. The largest number of genetic linkage analyses performed on any single cardiovascular trait in rats is systolic BP. Note in Fig. 1 that every rat chromosome has been reported to contain a BP QTL.

### STAGE 2: SUBSTITUTION MAPPING

Linkage analysis results in the identification of genomic regions in the range of 10–30 cM. A genetic interval of this size typically corresponds in humans and rodents to 10–30 Mb of DNA, or approx 100 to 300 genes (1), which is far too many candidates to begin functional evaluation of each gene individually. Thus, initial low-resolution linkage studies typically establish the map location to a resolution that is sufficiently precise to justify studies to confirm and define the genomic interval. In rodent model systems, this can be done with the use of consomic strains (8,9) and/or congenic strains (10). The use of consomic or congenic strains to confirm and define a QTL is known as substitution mapping. Congenic strains were first utilized by Snell (11). Developing congenic strains involves substituting a segment of chromosome, or in the case of a consomic, the entire chromosome, from one inbred strain (the donor strain) into another inbred strain (the recipient strain). This is done by crossing the donor and recipient strains to produce F1 animals, and then crossing F1 to recipient (first backcross). The offspring of this first backcross are genotyped using tail biopsy DNA for markers across a putative QTL containing region of a chromosome. Because chromosomal crossovers will occur between the donor and recipient chromosomes in the meioses of the F1 animal, recombinant chromosomes will be found in some of the offspring. This means that an animal with a specified donor chromosomal segment can easily be found. Such (heterozygous) offspring are backcrossed again to the recipient strain, and the offspring carrying the specific donor chromosomal segment are again selected. According to the classical approach, this procedure is repeated at least eight times. At each backcross, half of the unlinked heterozygous loci outside the selected (congenic) region become homozygous for the recipient allele; that is, the genetic background progressively becomes that of the recipient strain. After eight backcrosses, rats are selectively bred to fix the donor chromosomal



**Fig. 1.** Quantitative trait loci (QTL) for cardiovascular and related traits. QTL for cardiovascular and related traits that are cataloged by the rat genome database (RGD)(<http://rgd.mcw.edu/>) were searched in November 2003 and are plotted here against the rat chromosome number on which they are located. Bars represent the number of articles reporting QTL listed for each individual trait. \* Refers to refs. (83,84) that identified blood pressure (BP) QTL on the Y chromosome, which are not curated in the RGD. The 17 traits listed from the front row (foreground) to the back row are as follows: (1) left-ventricular mass; (2) mean arterial pressure; (3) renal damage; (4) renal disease; (5) aldosterone levels; (6) serum cholesterol levels; (7) serum triglyceride levels; (8) sensitivity to stroke; (9) obesity; (10) serum phospholipid levels; (11) hypercalcinurea; (12) atrial natriuretic peptide concentration; (13) vascular elastic tissue fragility; (14) cardiac mass; (15) proteinuria; (16) hypoxia-induced right ventricular hypertrophy; and (17) systolic BP (the tallest bars are on the back row).

segment in the homozygous state on the background of the recipient strain, producing a congenic strain. Congenic strains are indicated by the following notation: “Recipient strain. Donor strain,” wherein, “recipient strain” is the strain to whose genome a known genomic region from the “donor strain” is introgressed. For example, S.LEW refers to the introgression of a segment of LEW rats into the S-rat genetic background.

Recently, a technique called marker-assisted selection, or “speed congenics,” has enabled the development of a congenic strain using only 4–5 backcrosses (12–14). This technique involves selecting for the genome of the “donor” strain at the targeted chromosomal region, while simultaneously selecting against retention of the “donor” genome outside of the targeted chromosomal region using microsatellite markers throughout the genome.



There have been extensive linkage analyses performed in order to understand the genetic components of BP regulation, and for many of these studies congenic strains have also been developed to confirm and define the QTL (2, 12, 15–40). The origins and progress of most, if not all, of these studies were extensively reviewed by Rapp in 2000 (5). The present report focuses on progress during the last 3 yr that has been accomplished using substitution mapping as an approach to finding BP-altering genes.

## RESULTS

**Note:** Data for all the studies reviewed in this article are compiled in Table 1. For clarity of presentation, these results are categorized based on rat chromosome number. Data on congenic strains are emphasized.

### *Chromosome 1*

Initial observations with multiple genetic linkage analyses and subsequent substitution mapping approaches indicated the presence of at least one chromosomal region on rat chromosome (RNO)1 that influenced BP (5). Further substitution mapping from several groups over the past 3 yr revealed that RNO1 contains multiple BP QTL. Congenic substrains with relatively small regions of RNO1 have been developed and tested. These congenic strains include the transfer of Sabra hypertension-prone rat (SBH/y) into Sabra hypertension-resistant rat (SBN/y) and vice versa (32), Brown Norway (BN) into SHR (22), LEW into S (17, 21, 27, 41), SHRSP into WKY (33), SHR into WKY (35), WKY/Izm into SHRSP/Izm (36), and SHRSP/Izm to WKY/Izm (37) (Table 1). The locations of all the BP QTL that are confirmed by these substitution mapping studies are presented in Fig. 2A. It is evident from Figure 2A that these studies vary widely in terms of the resolution to which the locations of BP QTL have been determined. For example, Fig. 2A studies 1–4 have reported the localization of BP QTL to be within small regions from 2 to 20 Mb, whereas the majority of the other BP QTL represented in Fig. 2A are within large genomic regions of greater than 125 Mb. Of notable interest is also the fact that several of the BP QTL localized by these studies, with the exception of studies 1 and 6 (Fig. 2A), are clustered around the region of approx 100–225 Mb on RNO1. The present localization of BP QTL on RNO1 from multiple animal models is not sufficient to determine if allelic forms of the same gene(s) underlie the BP QTL found in each of the models.

### *Chromosome 2*

Following several linkage analyses using multiple F2 populations (reviewed in ref. 5), congenic strains introgressing the low BP QTL alleles from WKY or MNS into the S rat proved the existence of a substantial BP effector QTL on RNO2 (40). The congenic strain with the WKY donor was further genetically dissected by substitution mapping and reported to be clearly comprised of two BP QTL, QTL1 and QTL2 (29). These QTL are represented in Fig. 2B, studies 1 and 2. A third BP QTL called QTL3 was also suggested to be in the region represented in Fig. 2B, study 3 (29). However, experimental evidence for this QTL was weak (29). A congenic strain with the MNS donor on the S background localized a BP QTL to a 15-cM region (25). This BP QTL region is shown in Fig. 2B, study 4. In a second article published by the same group (42), the BP QTL was further localized to a 5.7-cM region on RNO2.

Table 1  
Status of Substitution Mapping for Blood Pressure Loci Using Congenic Strains

RNO no.	QTL study number	"From" marker	"To" Marker	Cytogenetic location of QTL	Name of the BP QTL	Congenetic strain (Recipient donor)	Reference
1	1	D1Rat211	D1Rat12	1p11	QTL2 region	S.LEW	41
1	2	D1Mgh7	D1Mco36	1q22	QTL1a region	S.LEW	27
1	3	D1Rat35	D1Rat131	1q22-32	QTL1b region	S.LEW	27
1	4	D1Wox34	D1Rat55	1q32-36	QTL1	SISA1	34
1	5	D1Rat56	D1Rat111	1q36-41	QTL2	WISA1	34
1	6	D1Rat10	D1Rat24	1p11-q12	SS1a	SBH.SBN	32
1	7	D1Rat27	D1Rat74	1q22-43	SS1b	SBH.SBN	32
1	8	D1Rat29	D1Rat57	1q22-q36	No name	WKY-1.SHRSP	33
1	9	D1Rat38	Mtpa	1q32-41	No name	WKY.SHR	35
1	10	D1Rat68	D1Rat71	1q41-43	No name	SHR.BN	22
1	11	D1Wox18	D1Wox10	1q22-43	No name	SHRSP/Izm.WKY/Izm	36
1	12	D1Wox18	D1Smu11	1q22-34	No name	WKY/Izm.SHRSP/Izm	37
1	13	D1Rat29	D1Rat57	1q22-43	No name	WKY.SHRSP	74
2	1	D2Rat35	D2Wox18	2q32	qtl1	S.WKY	29
2	2	D2Wox18	D2Wox25	2q34	qtl2	S.WKY	29
2	3	D2Wox25	D2Rat259	2q34	qtl3?	S.WKY	29
2	4	D2Rat166	D2Mgh10	2q32-34	no name	S.MNS	25
2	5	D2Rat21	D2Rat27	2q22	pBP1 QTL	WKY/Ij.SHR/Ij	43
2	6	D2Mgh10	D2Rat62	2q34-q43	pBP2 QTL	WKY/Ij.SHR/Ij	43
2	7	D2Rat40	D2Rat50	2q26	pBP3 QTL	SHR/Ij.WKY/Ij	43
2	8	D2Rat161	D2Mgh10	2q26-q34	dBQTL	SHR/Ij.WKY/Ij	43
2	9	D2Rat171	D2Arb24	2q24-42	no name	SHR.BN	38
2	10	D2Rat43	D2Mgh12	2q32-34	no name	SHRSP.WKY	44
3	1	D3Mco19	D3Mco24	3q42	minus BP QTL	S.R	19
3	2	D3Rat52	D3Rat130	3q12	minus BP QTL	S.LEW	56

(continued)

3	3	D3Chm63	D3Rat26	3q24	plus BP QTL	S.LEW	56
5	1	D5Uwm31	D5Rjr1	5q34	qtl1	S.LEW	30
5	2	D5Rat154	D5Wox39	5q32	qtl2	S.LEW	30
5	3	D5Wox20	D5Rat63	5q32-36	no name	SHR.BN	28
7	1	D7Mco19	D7Mco7	7q34	no name	S.R	31
8	1	Rbp2	D8Mit6	8q11-24	no name	SHR.BN-Lx	39
9	1	D9Mco14	D9Uia6	9q32-34	no name	S.R	68
10	1	D10Mco1	D10Wox23	10q26	qtl1	S.MNS and S.LEW	26
10	2	D10Mit1	D10Mco6	10q32.1	qtl2	S.MNS	26
10	3	D10M11Mit119	D10Rat27	10q26	qtl1	S.LEW	69
10	4	D10Rat204	D10Rat9	10q32.1	qtl2	S.LEW	69
10	5	D10Rat27	D10Rat93	10q26	qtl3	S.LEW	69
10	6	Myhse	D10Mit11	10q24-q32.1	BP/SP-1b	WKY.SHRSP	73
10	7	Myh3	Aldoc	10q24	BP/SP-1a	WKY.SHRSP	74
13	1	Consomic	Consomic	Not applicable	No name	S.BN	75
16	1	D16Rat21	D16Rat88	16p16	No name	S.LEW	76
19	1	D19Rat57	D19Mit7	19q12	SHR.BN-Agt	SHR.BN	78
20	1	D20Cebr215s7	D20Rat23	20p12	No name	SHR.1N	79
X	1	Ar-Mycs/Pfkb1	DXMgh3	Xp14-22	No name	BB/OK.SHR	80
Y	1	Consomic	Consomic	Not applicable	No name	SHR.BN	83
Y	2	Consomic	Consomic	Not applicable	No name	SHRSP.WKY and WKY.SHRSP	84

BP quantitative trait loci (QTL) that are reported in the literature are organized in this table by their location on the rat genome by chromosome. For clarity of presentation, a study number identifies each BP QTL. "From" marker indicates the marker that defines the p terminal end of the BP QTL region. "To" marker indicates the marker that defines the q terminal end of the BP QTL region. The cytogenetic location of each BP QTL was obtained by BLAST searching the rat genome using the "From" marker and the "To" marker at the website: [http://www.ensembl.org/Rattus\\_norvegicus/](http://www.ensembl.org/Rattus_norvegicus/). Whenever the markers were not listed at this website, alternate markers at the closest position on the radiation hybrid map were obtained from <http://rgd.mcw.edu/> and used instead for the BLAST search. The name of the BP QTL is entered as reported from the corresponding articles listed in the last column. A congenic strain is represented by names of two strains separated by a period. The recipient strain is the one to the left of the period, and the donor strain from which alleles are introgressed into the recipient genome background is shown to the right of the period.

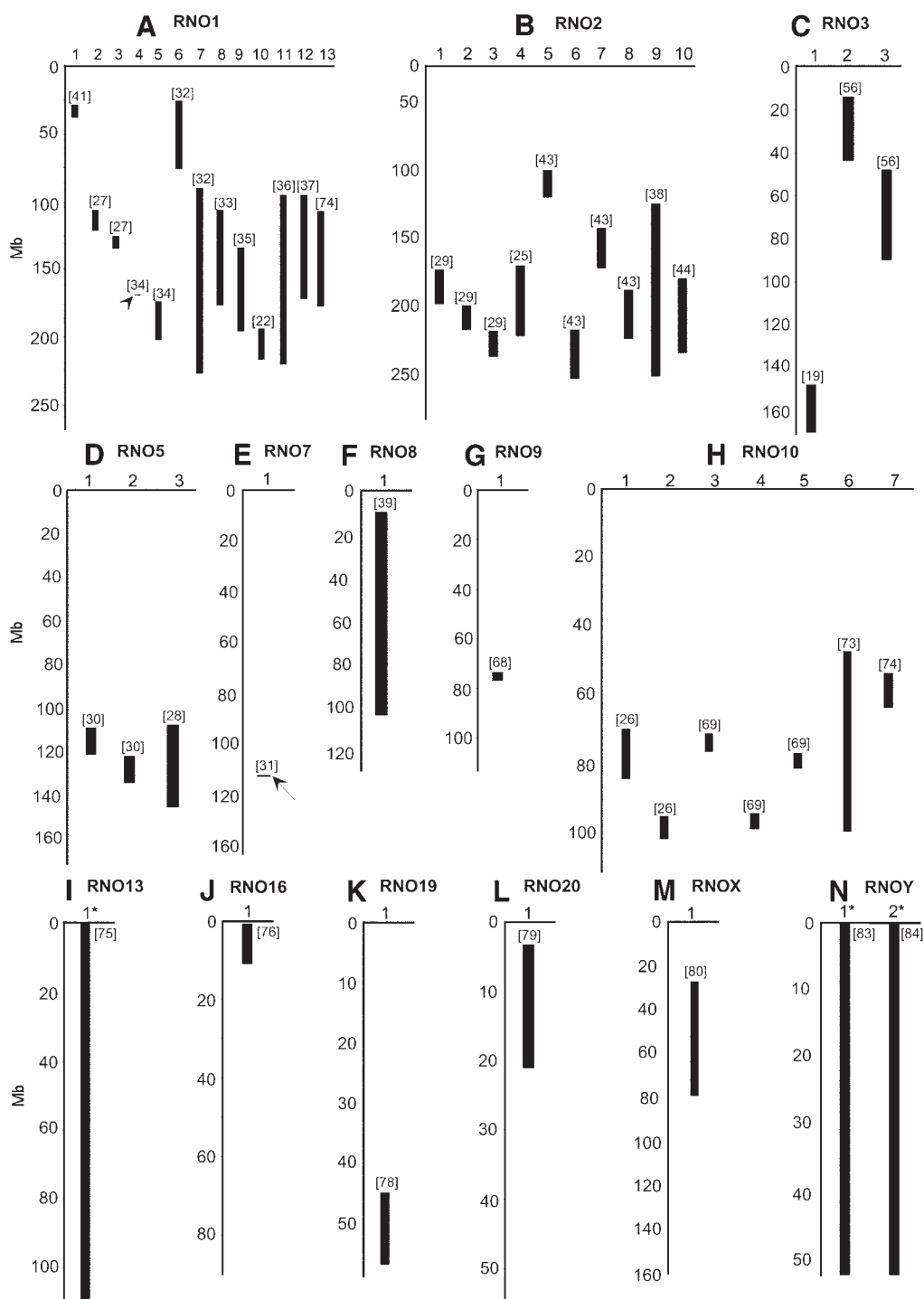


Fig. 2

BP QTL have also been localized on RNO2 using SHR as one of the parental strains. These earlier reports are reviewed elsewhere (5). More recently, Alemayehu and coworkers constructed and studied reciprocal congenic strains using SHR and WKY rats (43). This study resulted in the identification of at least four independent BP QTL (43). The locations of these BP QTL are shown on the physical map of RNO2 (Fig. 2B, studies 5–8). SHR alleles at three of the BP QTL shown in Fig. 2B, studies 5, 6, and 7, increase BP, whereas SHR alleles at the BP QTL shown in Fig. 2B, study 8, decrease BP. Alleles of BN rats from RNO2 introgressed into SHR to derive SHR.BN congenics are also reported to lower BP (38). As seen in Fig. 2B, study 9, the BP QTL detected in this study using SHR.BN congenic strain is large, and overlaps with most of the BP QTL localized by other substitution mapping studies. Taken together, all these results indicate that, like on RNO1, RNO2 is also rich in BP regulatory genes.

In addition to substitution mapping, McBride et al. have reported a study wherein they used microarray gene expression analysis as a complementary technique to detect superior candidate genes from among all the candidate genes within the BP QTL region (44) (Fig. 2B, study 10). Their study was aimed at identifying the gene(s) underlying the BP QTL on RNO2 that was previously defined by substitution mapping using SHRSP.WKY congenic strains (12,45). Genome-wide microarray expression profiling was undertaken to identify differentially expressed genes among the parental SHRSP, WKY, and SHRSP.WKY congenic rats. A significant reduction in expression of glutathione S-transferase type 1 (*Gstm1*), a gene involved in the defense against oxidative stress, was observed (44). More importantly, however, *Gstm1* mapped within the BP QTL region on RNO2. In the SHRSP rat, endothelial dysfunction has been attributed to increased generation of superoxide anions, which in turn leads to vascular oxidative stress (46–48). Induction of chronic oxidative stress by glutathione depletion can cause severe hypertension in normotensive rats (49). Based on these data the authors have concluded that *Gstm1* is a positional and physiological candidate gene for the BP QTL on RNO2 (44).

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**Fig. 2.** (continued from facing page) Schematic representation of locations of blood pressure (BP) QTL on the rat genome that are defined using congenic strains. Data presented in this figure are organized by rat chromosome numbers, i.e., each panel represents the BP QTL defined on one rat chromosome. The y-axis for each panel represents the length of the corresponding rat chromosome in megabases (Mb). The length of the rat Y chromosome was not available. Therefore, it was calculated based on the relative length of the human Y chromosome to the human X chromosome. Numbers denoted on the top of the figure represent study numbers assigned to each BP QTL in Table 1. Bars represent the locations of BP QTL that are defined by the “From” marker and “To” marker represented in Table 1. The locations of each of these markers on the physical map of the rat genome (v. 18.3.1; 4 November 2003) were determined by BLAST searching the Ensembl website ([http://www.ensembl.org/Rattus\\_norvegicus/](http://www.ensembl.org/Rattus_norvegicus/)) in November 2003. Whenever the markers were not listed at this website, alternate markers at the closest position on the radiation hybrid map were obtained from <http://rgd.mcw.edu/> and used instead for the BLAST search. Number in parenthesis on the top (or, in some cases, alongside) of each bar represents the number of the cited reference. Arrows in panels (A) and (E) represent BP QTL that have been localized to relatively small genomic intervals of less than 2Mb and 177kb, respectively. \* Indicates the study wherein a consomic strain is reported; microsatellite markers used to derive this consomic strain are not mentioned.

### ***Chromosome 3***

BP QTL on RNO3 are reported by multiple genetic linkage analyses (17,19,50–55). In 1999, Cicila et al. reported an S.R congenic strain that successfully “trapped” a BP QTL (19). As shown in Fig. 2C, study 1, this QTL on RNO3 is located toward the distal end of RNO3. However, it is only recently that Palijan et al. (56) constructed and studied multiple S.LEW congenic strains spanning the proximal region of RNO3, and reported that the proximal region of RNO3 contains a BP QTL (Fig. 2C, study 2) wherein alleles from the LEW rat lowered BP (56). In addition to these two BP QTL, a third BP QTL located towards the middle region of RNO3 (Fig. 2C, study 3) was also identified (56). The BP-enhancing effect of LEW alleles at this QTL was identified in S.LEW congenic rats, thereby demonstrating the utility of congenic strains for dissection and detection of closely linked alleles with opposing effects on BP.

### ***Chromosome 4***

Linkage analyses for BP identified QTL on RNO4 (57–60). A congenic strain containing a 12-cM region from the SHR rat introgressed into the BB/OK rat is also reported on chromosome 4 (61). Although these congenics provided strong evidence for the presence of genes on RNO4 involved in obesity and related phenotypes, the mean and diastolic BP of this congenic strain were not different from those in the parental BB/OK rat. Therefore, the existence of a BP QTL on RNO4 could not be confirmed using the BB.SHR congenic strain.

### ***Chromosome 5***

Garrett and Rapp (30) have developed and tested multiple S.LEW congenic strains on RNO5 and concluded that there are two closely linked interactive BP QTL on RNO5 (Fig. 2D, studies 1 and 2). The QTL was found to be interactive because strains with congenic segments that contained only QTL1 or QTL2 did not show a BP effect, but strains with congenic segments that contained both QTL did show a significant BP effect. BP QTL on RNO5 has also been localized using a SHR.BN congenic strain (Fig. 2D, study 3) (28). As seen in Fig. 2D, this BP QTL spans a large genomic interval that overlaps with both the QTL identified by Garrett and Rapp (30) using S.LEW congenic strains. It remains to be seen, however, whether the BP QTL colocalized to this region using SHR.BN congenic strain (Fig. 2D, study 3) is similarly comprised of multiple BP QTL that may interact.

### ***Chromosome 6***

No congenic strains for BP are reported on chromosome 6.

### ***Chromosome 7***

Substitution mapping for BP QTL on RNO7 (23,31) has historical significance for two reasons. First, unlike the other BP QTL that remain unidentified to date, the BP causative gene on RNO7 was suggested as early as the 1970s to be the steroid biosynthetic enzyme 11- $\beta$  hydroxylase (62). Substitution mapping pursued for the BP QTL on RNO7 can therefore be viewed as “a proof-of-principle” of the approach *per se*, as well as the actual identification of the BP causative gene. Secondly, the introgressed region (177kb) of the S.R causative gene constructed to define the BP QTL on RNO7 represents one of the best

“minimal” congenic strains ever constructed to date using rats (Fig. 2E) (31). This study therefore demonstrates that substitution mapping can indeed achieve the limit of resolution required to identify a candidate BP causative gene as one among five or six genes. As a result of compelling evidence supported by coding sequence mutations (23,63), and by functional studies on 11- $\beta$  hydroxylase (62,64,65), it was concluded that the gene responsible for the observed BP QTL on RNO7 in S rats is indeed 11- $\beta$  hydroxylase.

### *Chromosome 8*

Several independent genetic linkage studies provide suggestive evidence for BP QTL on RNO8. These have been reviewed previously (5). Two recent genetic linkage analyses using F2 (S  $\times$  SHR) and F2 (S  $\times$  AS) populations also indicate BP QTL on RNO8 (52,60). To date, very little progress has been made toward confirming and dissecting these BP QTL using substitution mapping. There has been one report of an SHR.Lx (Lx = BN.Lx/Cub) congenic strain that has successfully “trapped” a BP QTL in the region indicated in Fig. 2F (39).

### *Chromosome 9*

Genetic linkage analyses using F2 populations derived from (SHR  $\times$  WKY), (S  $\times$  R), and (S  $\times$  SHR) rats independently detected BP QTL on RNO9 (52,55,66,67). However, only the study involving the S and R has reported the confirmation of the BP QTL using S.R congenic strains (67). A recent study (68) has further localized this BP QTL to be within a relatively smaller genomic region of approx 2.4 cM on rat RNO9 (Fig. 2G). Localization of this BP QTL to this small interval has eliminated, among others, two solute carrier (Na<sup>+</sup>/H<sup>+</sup> exchanger) genes, *Nhe2* and *Nhe4*, as positional BP candidate genes. Yet another solute carrier (Cl<sup>-</sup>/HCO<sub>3</sub><sup>-</sup> exchanger) gene, *Ae3*, which could not be eliminated based on its location (*Ae3* is contained within the 2.4-cM BP QTL region), was however reasonably well-excluded as a BP causative gene based on the absence of sequence variants within the coding regions, and as a result of the lack of differential renal gene expression of *Ae3* between S and R rats (68).

### *Chromosome 10*

The search for BP causative genes on RNO10 began with several genetic linkage analyses detecting the presence of BP QTL on RNO10 (reviewed in ref. 5). Further linkage analysis indicated the presence of BP QTL on RNO10 in an F2 (S  $\times$  MNS) and F2 (S  $\times$  LEW) population, and initial congenic strains confirmed the presence of QTL with either MNS (16) or LEW (17) as the donor of large regions of RNO10. During the last 3 yr, multiple congenic substrains have been constructed and characterized for fine-mapping these BP QTL (26,69). These studies resulted in the localization of two RNO10 BP QTL, QTL1 and QTL2 (Table 1) to intervals of less than 2.6 cM and less than 3.2 cM, respectively (26). QTL 1 region (Fig. 2H, study 1) from the MNS congenic series also represents the QTL defined with the LEW congenic series. This implies that both LEW and MNS carry alleles that are functionally the same, but are in contrast with those in the S rat for BP QTL1. In contrast with QTL1, the QTL2 region (Fig. 2H, study 2) was identified in the congenic substrains derived from MNS, but not from LEW. Additional studies using S.LEW congenic substrains to localize BP QTL on RNO10 are recently reported (69,70). The method of measuring BP in these studies was by telemetry as opposed to the tail-cuff method used by Garrett et al. (26). Telemetry studies have cor-

robored the findings of Garrett et al. (26), and additionally resulted in further fine-mapping the QTL1 and QTL2 regions to approx 5.79 Mb and approx 4.75 Mb on RNO10 (Fig. 2H, studies 3 and 4). Also, this study (69) provided evidence for the existence of another BP QTL called as QTL3 (Fig. 2H, study 5), which is localized within the QTL1 region reported by Garrett et al. (26).

Linkage analyses in experimental crosses of SHRSP and WKY rats have suggested the presence of BP QTL on RNO10 in the region around the *Wnk4* (a serine/threonine kinase) locus. *Wnk4* has been implicated in human pseudohypoaldosteronism type II, which is a rare Mendelian form of arterial hypertension (71,72). WKY.SHRSP congenic strains constructed around the *Wnk4* locus were used to analyze the candidacy of *Wnk4* for BP control in rats (73). Whereas this congenic strain was proven to encompass a BP QTL represented in Fig. 2H, study 6, *Wnk4* was reasonably ruled out as a candidate gene for this QTL based on lack of mutations in the coding sequences and lack of differential renal gene expression of *Wnk4* between SHRSP and WKY rats (73). In another study by the same group using WKY.SHRSP congenic strains, a genetic interaction for the regulation of systolic BP has also been reported between the BP QTL on RNO10 shown in Fig. 2H, study 7 (74) and the BP QTL on RNO1 shown in Fig. 2A, study 13 (74). The congenic strain containing the BP QTL on RNO1 is reported in this study (74) to have no significant changes in systolic BP after salt loading compared with that in the parental WKY rats, but was reported to exert its effect only through interaction with RNO10. This is, however, the same congenic strain named WKY-1.SHRSP-Mt1pa/D1Rat200 (33), which was previously reported to have a significant systolic BP effect after salt loading (33) independent of RNO10. This inconsistency in the BP effect of the QTL on RNO1 renders the reported genetic interaction between the QTLs on RNO1 and RNO10 inconclusive.

### ***Chromosomes 11 and 12***

No studies using substitution mapping for identification of BP QTL have been reported on these chromosomes.

### ***Chromosome 13***

Since the review written by Rapp in 2000 (5), no additional congenic strains have been reported on this chromosome. Of related interest, recently a consomic strain constructed through the replacement of the entire RNO13 of the S rat with that of the BN rat was found to have markedly low BP compared with the S rat (Fig. 2I, study 1) (75).

### ***Chromosomes 14 and 15***

No congenic strains for BP have been reported on RNO14 or RNO15.

### ***Chromosome 16***

Suggestive evidence for linkage of BP to RNO16 on F2 (SHR × BN) (58) and F2 (S × LEW) (17) populations are reported. A recent report has confirmed the BP QTL on RNO16 using S.LEW congenic strains (76). This BP QTL is located towards the proximal region of RNO16, and is localized to approx 10 Mb region. Figure 2J represents the location of this BP QTL on the physical map of RNO16.



### ***Chromosome 17***

Two independent genetic linkage analyses using F2 (S × LEW) (17) and F2 (SBH × SBN) (77) rats detected BP QTL on RNO17. However, an S.LEW congenic strain did not corroborate the identification of the BP QTL found using the F2 (S × LEW) population. The reason for this remains unknown, although it was suggested that the BP QTL detected by linkage, could have been missed by the introgressed region on the congenic strain (5). Since then, there have been no additional studies for detecting or confirming BP QTL on RNO17 by substitution mapping. Therefore, conclusive experimental evidence for or against the existence of BP QTL on RNO17 is lacking.

### ***Chromosome 18***

There are no reports using substitution mapping for BP QTL on RNO18.

### ***Chromosome 19***

Linkage studies using the SHR have suggested BP QTL on RNO19 in the vicinity of the angiotensinogen gene (78). An SHR.BN congenic strain was constructed (78). This congenic strain significantly lowered BP compared with SHR parental. The introgressed region in this congenic strain (Fig. 2K) contains BN alleles at the angiotensinogen locus. Despite the BP-lowering effect observed, there were no major changes in plasma angiotensinogen or renin activities between this congenic strain and the parental SHR strain. The BP QTL “trapped” in this congenic strain is not explained by differences in plasma angiotensinogen levels or angiotensinogen expression.

### ***Chromosome 20***

Most of the substitution mapping studies for localizing BP QTL on RNO20 have focused on the major histocompatibility complex RT1 (reviewed in ref. 5). A recent study (79) reported the effect of high-fat diet on BP using SHR and SHR.1N (1N = BN.*Lx/Cub*) congenic rats. In these congenic rats, approx 30 cM region including the tumor necrosis factor (TNF)- $\alpha$  locus on RNO20 in the SHR rat was replaced by alleles from the BN.*Lx/Cub* rat (Fig. 2L). BN alleles in the introgressed region resulted in elevated BP compared with SHR rats. In this study, the authors were interested in examining the role of the TNF- $\alpha$  locus. Although they identified a number of sequence differences between SHR and SHR.1N rats in the regulatory regions of the TNF- $\alpha$  gene, no significant gene–diet interactions in mRNA expression were observed (79). TNF- $\alpha$  was therefore excluded as a candidate gene for the QTL on RNO20 that controls high-fat-diet-induced changes in BP.

### ***Chromosome X***

Evidence from a genetic linkage analysis using an F2 (SHRSP × WKY) population indicated the presence of a BP QTL on RNO X (6). In another F2 population derived from SBH and SBN rats (77), BP was linked to a broad region of RNO X overlapping with the region indicated by Hilbert et al. (6). Kloting et al. (80) constructed a congenic strain introgressing SHR alleles on the normotensive BB/OK background consisting of the genomic region indicated by Hilbert et al. (6) (Fig. 2M). This congenic strain had higher

BP compared with BB/OK. Since this study, there are no additional reports of substitution mapping for BP on RNO X.

### *Chromosome Y*

Previous studies indicated that hypertension in the SHR is linked to the Y chromosome (81,82). Replacing the SHR RNOY with the BN (= BN.*Lx/Cub*) RNOY resulted in significant decreases in systolic and diastolic BPs in the SHR.BN-Y consomic strain (83) (Fig. 2N, study 1). The effect of the SHRSP and WKY RNOY on BP was also studied using reciprocal consomic strains (84). Transfer of the Y chromosome from WKY onto SHRSP background significantly reduced systolic BP compared with BP of parental SHRSP (84) (Fig. 2N, study 2). In the reciprocal WKY.SHRSP consomic strain, systolic BP was increased compared with WKY parental strain (84). Based on these results, it was concluded that the Y chromosome of SHR and SHRSP models of hypertension harbors a locus contributing to systolic BP. In contrast, neither the origin of the Y chromosome nor the sex of the parental strain had any significant impact on the magnitude of the BP response to salt loading in the SBH model of hypertension (32).

## PERSPECTIVES

### *Finding BP Causative Genes by Substitution Mapping: Are We There Yet?*

Experiments conducted so far using substitution mapping to localize BP-controlling genes can be viewed as belonging to three categories: (1) construction and characterization of congenic strains with large introgressed segments (>20–30 cM)—such experiments are primarily focused on corroborating the initial BP QTL localization by genetic linkage analysis; (2) construction and characterization of congenic substrains to further localize a BP QTL within a smaller genomic region (<5–20 cM); and (3) construction and characterization of congenic substrains to fine-localize a BP QTL to a region less than 1 cM. Most of the studies conducted in the past 3 yr have advanced from category 1 to category 2. Very few, however, have progressed into category 3. So far, there is only one report that provides a reasonable “proof-of-principle” for substitution mapping using congenic strains as a correct approach for advancing from QTL localization to BP causative gene identification (31). Considering that evidence from genetic linkage analyses and substitution mapping indicate the presence of a large number of BP QTL (compiled at <http://rgd.mcw.edu/>) coupled with the fact that the identity of only one of them is known, an obvious question faced by investigators is whether to proceed with substitution mapping as a valid approach for finding the remaining BP causative genes. Two factors are in favor of searching for BP causative genes using substitution mapping.

1. *Sequencing of the rat and mouse genomes.* The major limitation in the construction of congenic substrains was that the development of microsatellite markers in a given target region was difficult. The availability of mouse and, subsequently, rat genome sequences has facilitated the identification of microsatellite markers in any targeted region of the rat genome, which can then be easily tested for polymorphisms and used to construct congenic strains. Additionally, now that the genome sequence is available and annotated for human, mouse, and rat genes, candidate genes in a QTL region can sometimes be found by searching a database.

2. *Microarray gene expression analysis.* Aitman et al. (85) successfully identified CD36 as a molecule responsible for insulin resistance in the SHR. The technique used by Aitman et al. (85) was to perform a comparative global gene expression analysis between a congenic strain with a relatively large introgressed region and one of the parental strains. The success of this study encourages the use of global gene expression analysis as a complementary technique to expedite BP causative gene identification using congenic strains. A recent study by McBride et al. has used gene expression analysis in combination with substitution mapping and identified glutathione *S*-transferase (*Gstm1*) as a BP causative candidate gene (44). Such reports do require additional compelling evidence to progress from the identification of a “BP causative candidate gene” to validation of it being “the BP causative gene.” For example, transgenic rescue experiments were used by Pravenec et al. (86) to establish unequivocally that CD36 is an insulin resistance QTL in the SHR.

To summarize, the recent reports discussed in this article indicate that it is possible to utilize substitution mapping as an efficient (albeit lengthy) strategy in not only confirming that genomic regions harbor BP QTL, but also defining with improved resolution the physical limits of the genomic regions surrounding each of these BP QTL. Further improving the resolution of localization by additional substitution mapping is, however, essential for advancing from BP QTL detection and confirmation to BP QTL identification. “Is it possible” to identify BP genes (10)? Yes. “Are we there yet?” No.

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## Local Production of Angiotensinogen

*Insights From Genetic Manipulation of Mice*

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*Kamal Rahmouni and Curt D. Sigmund, PhD*

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### SUMMARY

Genetic approaches such as gene targeting have been extensively used to gain a better understanding of the role of hormones and pathways involved in cardiovascular physiology. Given its pivotal role in cardiovascular homeostasis the renin–angiotensin system has been one of the most frequently targeted systems. Angiotensinogen (*AGT*), the only known precursor of the renin–angiotensin system, has received particular attention because genetic and molecular studies have shown that genetic variation at this locus impacts individual differences in blood pressure and the likelihood of developing essential hypertension. Knockout and overexpression of the *AGT* gene lead to adverse cardiovascular phenotypes. In this chapter we will review these data and detail a strategy using well-characterized cell-specific promoters to specifically target the *AGT* gene to defined cells and tissues. The development and characterization of these models has helped uncover the role and significance of local production of *AGT* in several tissues.

**Key Words:** Renin–angiotensin system; tissue angiotensinogen; blood pressure; transgenic mice; cell-specific targeting.

### INTRODUCTION

The renin–angiotensin system (RAS) is a major regulator of vascular hemodynamics and a determinant of cardiovascular homeostasis. It also contributes to the development and maintenance of various forms of hypertension and other cardiovascular diseases such

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as heart failure, cardiac hypertrophy, and atherosclerosis. Angiotensin II (ANG II), the primary effector of the RAS, is produced by the serial cleavage of angiotensinogen (AGT) by renin and angiotensin-converting enzyme (ACE). The actions of ANG II on distinct receptors (AT1a, AT1b, and AT2) located in vascular smooth muscle, kidney, adrenal gland, and brain lead to an elevation of arterial blood pressure through increased vascular resistance, cardiac output, sodium reabsorption and blood volume, and the production of other vasoactive hormones such as aldosterone (1,2). In addition, the RAS has been shown to play a role in regulating cell growth, in vascular smooth muscle proliferation, and during development (3,4). Based on the presence of different components of the RAS at both the protein and mRNA level in a variety of tissues, including adrenal gland, kidney, brain, heart, adipose tissue, and blood vessels, local-tissue RAS has been proposed (2,5,6).

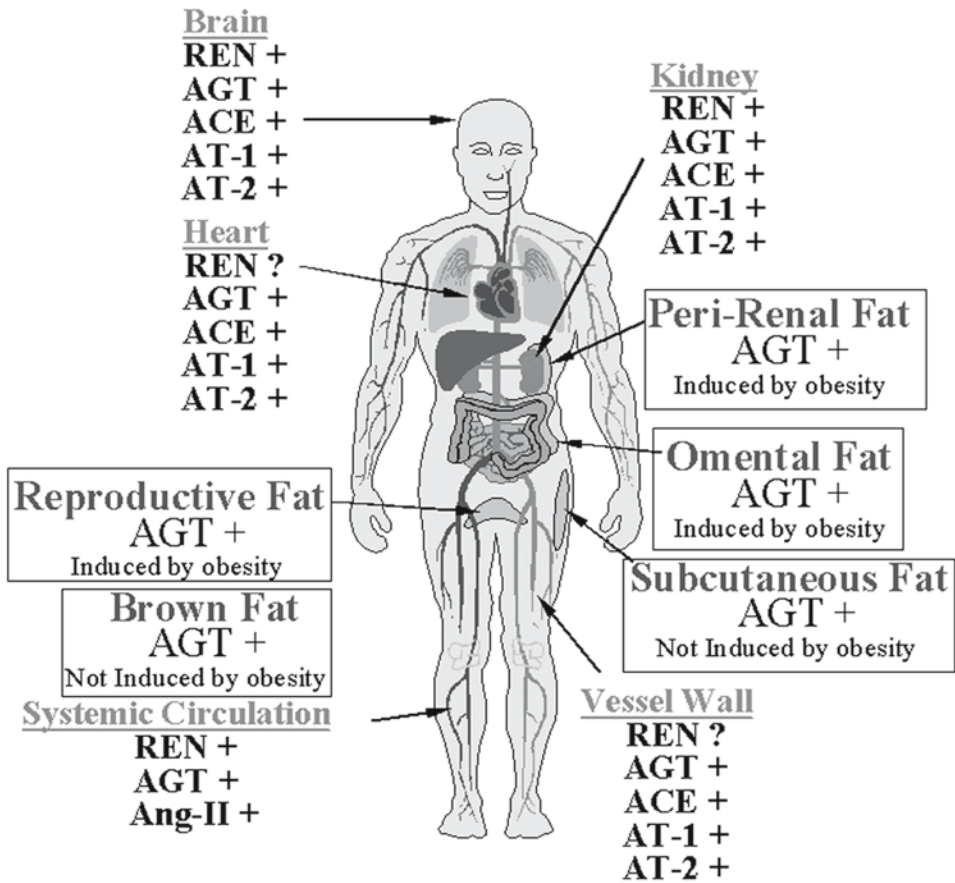
We will describe here some of the genetic manipulations used to study the role of AGT in the control of blood pressure and cardiovascular-related functions. AGT has received particular attention for many reasons. First, AGT is the only known precursor of ANG II. Second, the strongest evidence implicating a gene as a cause of human essential hypertension is for the *AGT* gene, and association of AGT levels and hypertension has been reported in families (7,8). Third, transgenic animals expressing high levels of AGT have elevated blood pressure (9), whereas knockout mice lacking AGT have low blood pressure (10,11).

### ANGIOTENSINOGEN (*AGT*) GENE

The human AGT mRNA is encoded from a gene located on chromosome 1, and contains five exons and four introns. The mRNA is 1455 nucleotides long and codes for a globular glycoprotein of 485 amino acids. The coding potential of AGT is located in exons 2 to 5, whereas the first exon encodes only a portion of the 5' untranslated region. Because the  $K_m$  of renin is close to the plasma concentration of AGT in humans, a modest change in its plasma concentration can affect the rate of ANG II formation. Mouse and rat renin hydrolyze a Leu–Leu bond in mouse and rat AGT, in contrast to the Leu–Val bond in human AGT. Because of this, and perhaps other differences in the AGT protein, the cleavage of AGT by renin is species-specific (12). Mice or rats producing human renin or human AGT, but not both, do not develop hypertension or any other phenotype (13,14).

Experimental evidence suggests that the liver represents the primary source of plasma AGT. AGT is probably not stored in hepatocytes, but is constitutively secreted into the systemic circulation (15). Production of AGT by the liver is regulated by several hormonal factors including estrogen, glucocorticoids, thyroid hormone, insulin, and ANG II (15,16). In accordance with the local-tissue RAS concept, AGT is synthesized in many tissues other than the liver, for example the brain, kidney, heart, adrenal gland, vasculature, and adipose tissue. The AGT produced by these tissues may be converted locally or released into the bloodstream to contribute to the circulating pool of AGT. The production of AGT in these tissues may also be regulated locally, independently of the circulating system. The presence of other components of the RAS, such as renin and ACE necessary for the biosynthesis of active angiotensin peptides in most of the tissues that produce AGT, provides further evidence for the *de novo* synthesis of ANG II in these tissues (Fig. 1). However, the existence of alternative pathways for the production of ANG II in tissues where some components of RAS were not found cannot be excluded (6).





**Fig. 1.** Different sites of expression of the components of the renin–angiotensin system (RAS) in the body. The classical systemic RAS in which angiotensinogen (AGT) is derived from the liver, renin from the kidney, and angiotensin-converting enzyme from lung is shown. Presence of different components of the RAS in a variety of tissues has led to the concept of local RAS. AGT expression is also detected in adipose tissue and is induced by obesity in some depots. Adipose tissue is becoming recognized as another potential tissue RAS.

## TARGETING SYSTEMIC AGT

In order to evaluate the effect of *AGT* gene disruption on blood pressure regulation, conventional gene targeting in embryonic stem cells was used to delete the *AGT* gene (10,11). As expected, homozygous *AGT* knockout mice had no detectable plasma AGT whereas there was a 58% decrease in heterozygous mutant mice. Survival studies have shown that absence of a functional *AGT* gene is compatible with survival to birth, but postnatal survival is severely compromised, perhaps because of the altered renovascular development in these mice (17). Furthermore, compared to wild-type mice, knockout of the *AGT* gene resulted in a marked decrease in blood pressure.

Further evidence for the crucial role of AGT in blood pressure regulation is derived from studies of mice with different copies of the *AGT* gene (11). Plasma AGT levels increase with the *AGT* gene copy number (by 35% in 1 copy, 124% in 3 copies, and 145% in 4 copies). Interestingly, blood pressure increased in proportion to the increase in *AGT*

gene copy number, averaging 8 mmHg per copy. This experiment demonstrates clearly that variation in circulating AGT can affect blood pressure. It also establishes the potential for an effect on blood pressure by genetic influences arising from differences in circulating AGT.

We generated transgenic mice containing the entire *hAGT* gene in order to study its tissue- and cell-specific expression and its role in the pathogenesis of hypertension (14). The transgene employed contains all five exons and intervening intron sequences, and extends approx 1.2 kb upstream and 1.4 kb downstream of the gene. The expression pattern of *hAGT* gene was found to be very consistent with the tissue-specific expression of the mouse and rat *AGT* genes and with the expected pattern of AGT expression in humans. Consistent with previous reports, analysis of the cellular origin of kidney hAGT in these transgenic mice revealed that it was exclusively localized to the epithelial cells of the proximal convoluted tubules (18). All together, these data demonstrated that the *hAGT* transgene was appropriately regulated. It also suggests that these transgenic mice represent a valid model for examining the regulation of *hAGT* gene.

The hAGT released into the plasma of the transgenic mice was determined to be functional because infusion of a single bolus dose of purified human renin resulted in transient increase in blood pressure of approx 30 mmHg within 2 min. Furthermore, crossbreeding of these mice expressing hAGT with transgenic mice containing a genomic clone encoding the human renin resulted in chronic elevation of blood pressure (19). These double-transgenic mice also had an altered baroreflex response (i.e., resetting of the arterial baroreflex of heart rate to a higher pressure without significantly changing the gain or sensitivity of the reflex).

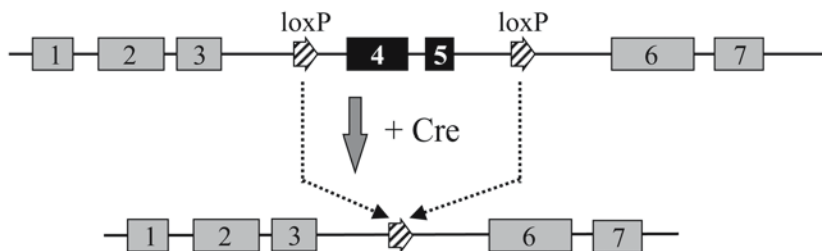
We also evaluated whether elements of the human RAS could functionally overcome the hypotension, renal pathology, and reduced survival observed in the AGT knockout mice. This was achieved by breeding the double-transgenic (hAGT/human renin) mice with the AGT knockout mice (20). We found that the presence of both human transgenes rescued the postnatal lethality in AGT knockout mice. The lower blood pressure and renal lesions observed in AGT knockout mice were also corrected by the presence of the human transgenes. These findings indicate that it is the absence of ANG II, and not AGT *per se*, that is responsible for the abnormalities observed in the AGT mutant mice. These data demonstrate also the ability of human renin and *AGT* genes to replace the mouse *AGT* gene.

## TARGETING THE TISSUE AGT

In recent years there has been a refocusing of effort on understanding the role of tissue RAS in the regulation of blood pressure and fluid and electrolyte homeostasis, as well as their involvement in cardiovascular and renal diseases. The advances in genetic manipulation, which allow gene ablation and tissue-specific gene targeting, have provided a new set of tools to experimentally dissect the role of the AGT produced in different tissues. We will illustrate the important role of local AGT synthesis by describing the development and characterization of mouse models in which AGT has been targeted to specific cell types.

### *Liver AGT*

As mentioned previously, the liver is considered the primary site of AGT synthesis and the main source of plasma AGT. We therefore examined the effects of hepatic AGT elimination on the circulating pool of AGT. To knock out AGT specifically in the liver,



**Fig. 2.** Schematic of Cre-loxP System. The targeted gene is designed with loxP sites flanking one or more important coding exons (filled boxes). The addition of Cre-recombinase will result in the deletion of the flanked exon, disabling the gene. (For more details, *see* ref. 56).

we used the Cre-loxP system (21) (Fig. 2). We designed a transgene in which exon 2 of the hAGT gene is flanked by loxP sites (22). Efficient recombination was observed in transgenic mouse liver upon intravenous administration of adenovirus that expressed Cre recombinase (Adcre). Systemic administration of Adcre caused hepatic-specific deletion of hAGT, as no hAGT mRNA or protein was detected in the liver, whereas in the kidney its expression was unaltered. This is consistent with the results demonstrating that intravenous administration of adenoviruses affects primarily the liver, but not other tissues (23). Further analyses have shown that the deletion of the *hAGT* transgene in the liver was associated with a significant decrease in the plasma level of hAGT (over 90% of control levels) and a markedly attenuated or absent pressor response to infusion of purified recombinant human renin protein. These results strongly suggest that the liver is by far the main source of AGT. A potential limitation of this Cre-loxP system is its effectiveness for acute, but not chronic, elimination of hepatic hAGT. Indeed, transgenic mice containing the floxed hAGT construct exhibited transient lowered blood pressure in response to Adcre, likely a result of repopulation of the liver with AGT-positive cells (24). Chronic elimination of hepatic hAGT will likely require the use of transgenic mice endogenously expressing Cre recombinase in the liver (25).

### *Renal AGT*

In the kidney, the proximal tubule is the major site of angiotensin production (18). The AGT mRNA is localized predominantly in the proximal renal tubule, with considerably lesser amounts in distal tubular segments and glomerular tufts. AGT and ANG II have been detected in proximal tubule fluid (26,27), and are secreted through the apical membrane in proximal tubule cells in culture (28). The presence of AGT in the urine is an indication of its secretion in the tubular fluid and its transition through the entire nephron. However, it was not clear whether the AGT produced locally in the kidney contributed to the circulating pool of this protein. A role for intrarenal AGT in the regulation of blood pressure and renal function was also hypothesized. This is based on the finding that the kidney contains not only the AT1 and AT2 receptors, but also all of the enzymes required to convert AGT to the active hormone ANG II.

To address the potential role of AGT synthesized within the kidney, we produced transgenic mice with a kidney-specific expression of AGT. This was achieved by designing a transgene that contained the *hAGT* gene driven by the kidney androgen-regulated protein promoter (29–31). This promoter was chosen because, like AGT, it is expressed in the renal proximal tubule cells and is responsive to androgens (32). No hAGT was

detected in the plasma of the transgenic mice, which suggests that the kidney does not contribute to the circulating pool of AGT. However, elevated concentrations of hAGT were clearly observed in the urine of the transgenic animals, confirming its release in the tubular lumen. To test the impact of the intrarenal production of AGT on blood pressure, we bred these transgenic mice with the mice that express systemic human renin. Double-transgenic male mice had increased blood pressure but normal circulating ANG II levels (30). The minimal blood pressure response to the acute intravenous infusion of the selective AT1 receptor antagonist losartan in these double-transgenic animals as compared with the systemic model confirms that the elevation in blood pressure is a result of the activation of a selective intrarenal mechanism. Our data support the conclusion that kidney AGT participates in the control of blood pressure and renal homeostasis, suggesting that a dysfunction in the intrarenal production of AGT could likely contribute to hypertension. Furthermore, the usefulness of the kidney androgen-regulated protein promoter to study the kidney AGT illustrates the possibility of specifically targeting the intrarenal systems using gene overexpression.

### ***Brain AGT***

The brain RAS has received much attention recently because accumulated experimental evidence has shown its involvement in the development of different forms of hypertension (33). The central action of ANG II is known to regulate several activities that can affect blood pressure, including sympathetic nervous function, vasopressin release, drinking behavior, and the appetite for salt. The expression of AGT is widely distributed throughout the brain, with high expression in those areas involved in cardiovascular regulation, such as the hypothalamus and preoptic areas (34). Renin is also thought to be expressed in the brain and appears localized primarily in neurons but also in some glia (35,36), although this is controversial. To experimentally dissect the role of AGT produced in the brain we developed transgenic mice models that express hAGT in the brain, driven by different promoters. Given that astrocytes are considered as the major source of brain AGT (37), we first generated transgenic mice that express hAGT under the control of the glial-specific, glial fibrillary acidic protein (GFAP) promoter (38). In these mice, hAGT protein was mainly localized in astrocytes, but was also present in neurons in the subfornical organ. Measurement of circulating hAGT has shown that plasma levels of hAGT protein were not elevated over baseline. Direct administration of human renin in the cerebral ventricles of these transgenic mice caused a dose-dependent increase in blood pressure, which was prevented by pretreatment with an AT1 receptor blocker. Furthermore, crossing the GFAP-hAGT mice with mice that express human renin systemically led to the development of mice that exhibited moderate, but chronic elevation in blood pressure (about 15 mmHg). In further studies we found that when these double-transgenic mice were provided a choice between tap water and saline, they exhibited a significantly greater preference for saline, because they drink nearly 40% of their total volume as saline as compared with 26% in control mice. To ascertain that the phenotype observed in these double-transgenic mice is a result of the ANG II produced locally in the brain, we crossed the GFAP-hAGT mice with transgenic mice that express human renin under the control of this GFAP promoter (39). The double-transgenic animals had an increase in blood pressure, drinking volume, and salt intake, which indicates that these alterations are caused by the local production and action of ANG II in the central nervous system. All together, these data demonstrate that the AGT produced by astrocytes plays

a major role in blood pressure regulation and fluid homeostasis. Other models using antisense techniques to reduce glial AGT similarly support its importance in blood pressure regulation (40), although this is beyond the scope of this chapter. Interestingly, and perhaps surprisingly, glial-specific expression of ANG II was reported to correct the renal defects observed in AGT knockout mice (41).

Because both in vitro and in vivo studies have shown that neurons also produce AGT, which is in accordance with the status of ANG II as a neurotransmitter, we developed mice that express AGT driven by synapsin I (SYN I), a neuron-specific promoter (42). In these transgenic mice, the hAGT protein was detected exclusively in neurons. However, the widespread expression of hAGT throughout the central nervous system that was observed in these animals does not follow the pattern normally exhibited by AGT. This is an experimental limitation resulting from the absence of an appropriate promoter that would allow us to specifically target only those neurons normally expressing AGT, such as the subfornical organ. The hAGT produced in the brain is active as demonstrated by the dose-dependent response of blood pressure to central injection of purified human renin in these transgenic mice. A moderate chronic hypertension and increased drinking intake and salt preference were obtained when these SYN I-hAGT mice were bred with mice that express the human renin driven by the same promoter (39). These findings indicate that neural-derived AGT can be converted locally to ANG II, acting as an autocrine and/or paracrine effector to alter blood pressure and the equilibrium of fluid and electrolytes. Although similar phenotypes regarding cardiovascular function and fluid-electrolyte homeostasis were obtained in both of our transgenic models (whether the expression of hAGT is driven by GFAP or SYN), further studies are required to examine the consequences of differential expression of hAGT on several other functions in which brain RAS is involved, such as cognition, memory, pain perception, sexual behavior, and stress. Models expressing AT-1 receptors in the brain driven by the rat neuron-specific enolase promoter have also been recently reported (43).

### *Cardiac AGT*

The identification of a local RAS in the heart has led some to speculate that its activation could lead to ventricular hypertrophy and myocardial fibrosis independently of blood pressure. Although the source of cardiac renin (de novo local synthesis vs uptake from the circulation) is still debated, strong evidence supports an intracardiac production of ANG II (44). AGT mRNA and protein has been detected in all parts of the hearts of different species, as well as in cultured cardiac myocytes and fibroblasts (44,45). The cardiac production of AGT seems to be regulated by different stimuli, such as glucocorticoids,  $\beta$ -adrenergic receptor agonists, and stretch (45). Furthermore, the levels of cardiac AGT mRNA were significantly increased in hypertensive rats (46), but also in patients with ventricular hypertrophy and heart failure (47).

In order to examine the consequence of increased local production of AGT on cardiac function, Mazzolai et al. (48) produced mice overexpressing the rat AGT gene specifically in the heart. The cardiac-specific  $\alpha$ -myosin heavy-chain promoter was used to direct AGT synthesis in cardiomyocytes. Cardiac specificity of the transgene was demonstrated by its expression in the heart, but not in the other tissues tested. The increase in AGT production in the heart of transgenic animals resulted in increased cardiac ANG II. Cardiac hypertrophy, characterized by the presence of enlarged cardiomyocytes, was observed in all transgenic mice independently of hypertension. However, no significant

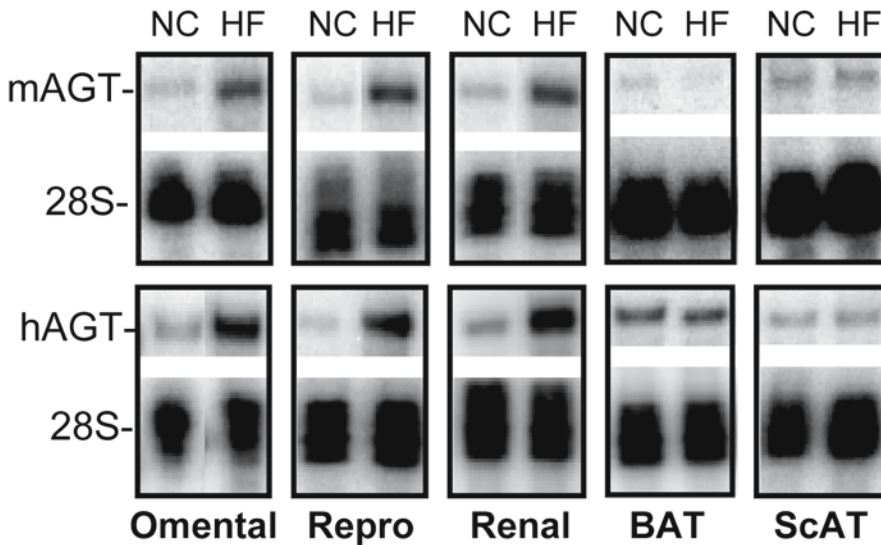
development of fibrosis was observed in these transgenic animals. These data underline the pathophysiological role of increased AGT synthesis in the heart, which appears to promote cardiac hypertrophy. The direct targeting of angiotensin peptides to the heart using a novel fusion protein has also been shown to induce the expression of atrial natriuretic peptide mRNA, a marker of cardiac hypertrophy (49).

To further examine the role of heart AGT in cardiac hypertrophy, Kang et al. (50) used an approach involving the re-expression of AGT in liver and brain, but not heart, in AGT knockout mice. This was achieved by breeding AGT-deficient mice with transgenic mice expressing rat AGT only in the liver and brain. The resulting animals have increased blood pressure, and rescued the developmental defects in the kidney of AGT knockout animals. Interestingly, the mice with AGT only in the brain and liver developed less cardiac hypertrophy than mice capable of making AGT in the heart. The perivascular and interstitial fibrosis was also less pronounced in the mice lacking AGT only in the heart. These data demonstrate that locally synthesized AGT is important for cardiac damage. However, the fact that mice with blunted cardiac AGT synthesis still develop cardiac hypertrophy and fibrosis suggests that uptake of the AGT or other angiotensin peptides from the circulation may also be very important.

### *Adipose Tissue AGT*

Until recently, adipose tissue was considered exclusively as a body energy store without other function. However, the realization that adipocytes can produce many peptides and hormones including leptin, atrial natriuretic peptide, and AGT has led us to view this tissue as an endocrine secretory organ with consequences on the cardiovascular system (51). For instance, AGT produced by the adipose tissue may act locally to affect adipocyte growth and differentiation, or may be secreted into the bloodstream, thereby contributing to the circulating pool of AGT. Synthesis of AGT in adipose tissue associated with the presence in this tissue of the enzymatic cascade and ANG II receptors makes possible the local production and action of ANG II (52,53). To study the specific role of the adipose AGT on fat mass and blood pressure, Massiera et al. (54) have generated transgenic mice in which AGT is restricted to adipose tissue. This was achieved by developing mice that express a transgene containing the full-length rat AGT cDNA driven by the adipocyte-specific aP2 promoter. These transgenic mice were then back-crossed with AGT knockout mice to generate animals whose AGT expression is restricted to adipose tissue. Compared with AGT knockout mice, which have no detectable AGT in the plasma, the circulating levels of AGT in the transgenic mice expressing AGT only in the adipose tissue was about 20–30% of the wild-type mice. It is noteworthy to mention that the analysis of AGT secretion from adipose tissue explants has shown an approximately ten-times higher production of AGT in the transgenic mice than wild-type animals, partially explaining the relatively high plasma AGT levels detected in the mice. Nonetheless, expression of AGT only in the adipose tissue rescued the hypotensive phenotype as well as the renal dysfunction observed in the AGT knockout mice.

This demonstration that AGT produced by the adipose tissue may be released in the bloodstream suggests that the high circulating levels of AGT associated with obesity may be a result of the increased fat mass. We have recently tested the hypothesis that obesity may be associated with an adipose tissue-specific increase in the *AGT* gene transcription (summarized in Fig. 1) (55). We found that mice with obesity induced by high-fat diet exhibited a greater *AGT* gene expression in visceral fat (omental fat, perirenal fat, repro-



**Fig. 3.** Effect of the obesity induced by 20 wk of high-fat diet on the expression of mouse angiotensinogen (*AGT*) gene (*mAGT*) in wild-type mice (top) and *hAGT* in the transgenic mice (bottom) in the intraabdominal fat depots. The effect of high-fat diet (HF) was compared to normal chow (NC) as a control. *mAGT* and *hAGT* mRNA expression was quantified using RNase protection assay and the 28S rRNA was used as an internal control. Repro, reproductive fat; Renal, peri-renal fat; BAT, brown adipose tissue; ScAT, subcutaneous adipose tissue. (Reprinted from ref. 55, with permission from the American Physiological Society.)

ductive fat: epididymal fat in males and parametrial fat in females, Fig. 3). However, the expression of the *AGT* gene was not affected by high-fat diet in nonadipose tissues (liver, kidney, and heart), in brown adipose tissue, or in subcutaneous fat. To study the effect of diet-induced obesity on *hAGT* gene expression, we took advantage of the transgenic mice that express the *hAGT* gene. A similar pattern of response was observed for the *hAGT* gene when obesity is induced in these transgenic animals by high-fat diet (Fig. 3), suggesting that the endogenous mouse *AGT* and transgenic *hAGT* are regulated in the same fashion. Our findings demonstrate that 1.2 kb of regulatory sequences present in the *hAGT* transgene is sufficient to transcriptionally respond to a high-fat diet, and in an adipose-specific manner. All together, these data indicate that *hAGT* transgenic mice provide a unique model for studying the regulation of *hAGT* gene in obesity.

## CONCLUSION

Gene targeting in the mouse has yielded remarkable advances in our understanding of the role played by specific genes in the regulation of the cardiovascular system. The greatest limitation of this strategy is the lethal phenotype that results from the lack of proteins, such as *AGT*, serving essential functions. Furthermore, such strategy does not distinguish between the role of an endocrine system and tissue-restricted biological systems. Differentiating between these systems can be technically challenging because of the specificity of pharmacological inhibitors or difficulties in their delivery to appropriate cell types. The strategy of cell-specific targeting coupled with new advances in genetic methodology allowing an investigator to regulate transgene expression or generate cell-

specific knockouts is a valid alternative through which to distinguish and elucidate the physiological role of different gene products. We have illustrated that it is feasible to target different locations of AGT to evaluate the contribution of each site of production of this hormone precursor *in vivo*.

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## Cardiovascular Pharmacogenomics

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### SUMMARY

Pharmacogenomics is a field aimed at understanding the genetic contribution to inter-patient variability in drug efficacy and toxicity. The promise of pharmacogenomics is that it will allow for individualized therapy based on a person's genetic makeup, allowing for the selection of the drug that is likely to be the most effective with minimal risk of toxicity. Pharmacogenomics can also be used to help guide the drug discovery process, although this is not discussed in this chapter. This chapter describes the various manners in which pharmacogenomics might be used in the treatment of cardiovascular disease, including those whose treatment is by trial-and-error (like hypertension), and those whose treatment is protocol-driven (like heart failure or myocardial infarction). The current literature on genetic associations with efficacy and toxicity of cardiovascular drug response is reviewed. The approaches to pharmacogenomics research are also reviewed, including a candidate gene-driven approach and genomic approaches. There is discussion of how the approaches to date, usually single-candidate gene, must become more sophisticated in order to better understand the genetic basis for variable drug response. There is also extensive discussion of the various laboratory approaches that are essential to pharmacogenomics, including a number of different polymorphism genotyping methods. Pharmacogenomics holds great promise for enhancing our understanding of cardiovascular drug response and allowing for individualized therapy. There will be substantial work in this field in the coming years, which will hopefully lead to the point of individualization of cardiovascular drug therapy in patients.

**Key Words:** Pharmacogenetics; pharmacogenomics; drug metabolism; pharmacology; single nucleotide polymorphisms; genotyping methods.

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## INTRODUCTION

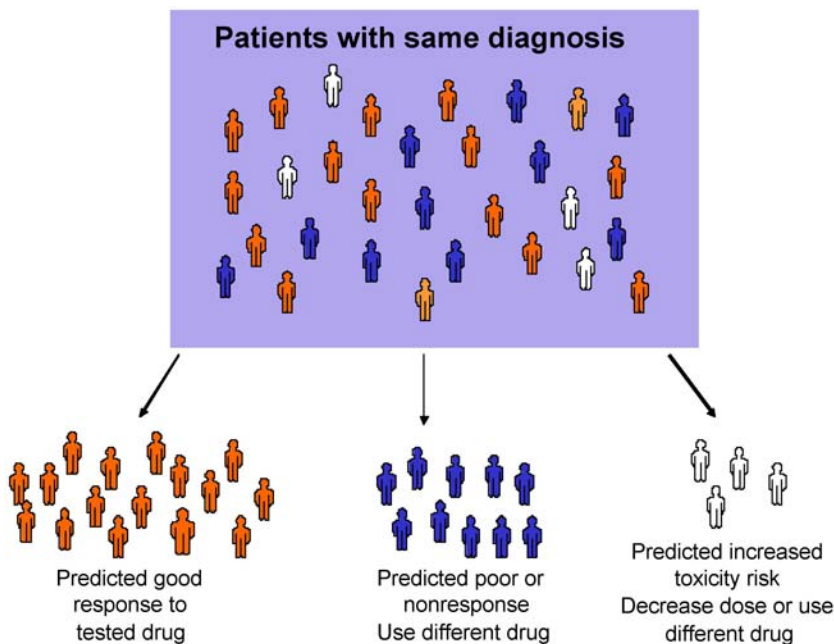
It is well-recognized that there is great interpatient variability in response to drugs, including drugs used in the treatment of cardiovascular disease. Whereas some patients may attain the desired therapeutic response from a given drug, others may show no therapeutic benefit, and others may experience toxicities. Currently, there are limited, if any, tools available to the clinician to help him or her predict into which of these categories a patient might fall if that patient is prescribed a specific drug therapy. There are numerous factors that influence the variability in response to a drug, including the patient's age, race, concomitant diseases, interacting drugs, and renal and hepatic function. However, there is increasing evidence that for a number of drugs, genetic variability plays an important (and sometimes central) role in variable response to drugs. Thus, the field of pharmacogenomics is focused on providing an understanding of the genetic contribution to variable drug response. There is some debate about the differences in definitions of the terms "pharmacogenetics" and "pharmacogenomics," but such discussions seem to have little value. If one chooses to give them different definitions, pharmacogenomics is the broader term, which encompasses pharmacogenetics. As such, we will only use the term pharmacogenomics herein.

There are two broad manners in which pharmacogenomics can be approached from a research perspective. The first is to use genomic information to help identify potential new drug targets. The concept is that if the genetic or genomic basis of a disease is understood, then it might be possible to develop highly targeted therapies. Currently, all drugs used today represent only about 500 different drug targets, although it is estimated that there are probably 5000 to 10,000 potential drug targets in the body. Thus, such an approach clearly has the potential to increase the number of novel drug targets. Although there are certain examples in the area of cancer of marketed drugs that have been developed through such an approach, it would be hard to argue that any marketed cardiovascular drugs have been discovered by this strategy. Because many of the other chapters in this text will deal with ways in which genomics technologies can be useful in understanding cardiovascular disease and therefore in identifying potential new drug targets, we will not focus on these issues in this chapter.

The other manner in which pharmacogenomics can be approached is through attempts to elucidate the genetic (or genomic) contribution to variable drug response of existing drugs, either those on the market or currently under development. The bulk of this chapter will focus on this type of research effort. We will summarize the current literature in the area of cardiovascular pharmacogenomics, discuss the various methodological approaches that can be taken, and review relevant laboratory techniques.

### *How Pharmacogenomics Might Be Used Clinically*

Pharmacological management of cardiovascular diseases is largely based on evidence from clinical trials and guidelines from expert consensus panels. For example, current consensus guidelines recommend the institution of angiotensin-converting enzyme (ACE) inhibitors and  $\beta$ -blockers and consideration of spironolactone in all patients with severe heart failure, based on evidence that these drugs reduced morbidity and mortality in several large clinical trials (1). However, not all participants enrolled in heart failure studies with ACE inhibitors,  $\beta$ -blockers, and spironolactone derived clinical benefits from these drugs, and some study participants experienced serious adverse drug reactions requiring study discontinuation. For instance, in the Studies of Left Ventricular Dysfunc-



**Fig. 1.** Clinical potential of pharmacogenetics. Patients with the same empirical diagnosis (e.g., hypertension, leukemia, etc.) are typically treated in the same manner, although their responses to drug therapy will not be the same. Pharmacogenetics has the potential to provide a tool for predicting those patients who are likely to have the desired response to the drug, those who are likely to have little or no benefit, and those at risk for toxicity. This would allow tailored therapy that should reduce adverse reactions to drugs, and increase efficacy rates. (Reproduced with permission from ref. 64.)

tion (SOLVD), one of the first trials to demonstrate the benefits of ACE inhibitor therapy in heart failure, approx 5% of participants discontinued ACE inhibitor treatment because of worsening disease (2). An additional 8% of participants discontinued treatment because of adverse drug effects. Thus, one cannot assume that a given patient will have a favorable response to a particular drug, despite benefits observed in the overall population of a clinical trial.

Pharmacogenomics might enable clinicians to individualize cardiovascular drug therapy based on a person's genotype, as shown in Fig. 1. This approach to cardiovascular disease management has the potential to streamline the management of diseases such as heart failure. Rather than starting all heart failure patients on the same therapy, drug regimens might be tailored according to each individual's genetic predisposition for obtaining benefit or experiencing harm from a particular drug. For instance, patients with heart failure would be genotyped for drug response at the time of heart failure diagnosis. Then, the drug or drugs expected to produce the greatest clinical response for a particular patient with regard to reductions in morbidity and mortality would be started and titrated to maximally tolerated doses. This approach would be particularly beneficial for patients with inadequate blood pressure to safely take recommended doses of both ACE inhibitors and  $\beta$ -blockers. Patients with a genetic predisposition to serious adverse drug effects, such as angioedema from ACE inhibitors, could be started on alternative therapy. Not only would this approach to heart failure management

potentially improve clinical outcomes in the heart failure population as a whole, but it might also reduce healthcare costs associated with the management of adverse drug reactions and with unnecessary polypharmacy.

Pharmacogenomics also has the potential to eliminate the trial-and-error approach to the management of diseases such as hypertension. Patients with hypertension are currently started on one or two drugs to lower their blood pressure. Therapy is then continued as initially prescribed if an adequate response is obtained, or, more commonly, therapy is adjusted until there is sufficient blood pressure reduction. Often, initial drug therapy is abandoned because of a lack of response or intolerable adverse effects, and alternative agents are instituted. For example, in the Antihypertensive and Lipid-Lowering Treatment to Prevent Heart Attack Trial (ALLHAT), 15–20% of participants discontinued initial antihypertensive therapy, usually as a result of adverse drug events or therapeutic failure (3). With pharmacogenomics, clinicians might be able to predict a patient's response to various antihypertensive medications prior to drug initiation and then prescribe the drug expected to produce the greatest blood pressure response with the least potential for harm. Such an approach to hypertension management may shorten the duration until blood pressure control is achieved and thus the length of time the patient is at risk for hypertension-related end-organ damage. Additionally, this approach would reduce the number of office visits required to find the best therapy, minimize exposure to agents that will not provide benefit but could still cause adverse effects, and avoid costs for ineffective agents and the adverse events they might cause.

## CARDIOVASCULAR PHARMACOGENOMICS: CURRENT EVIDENCE

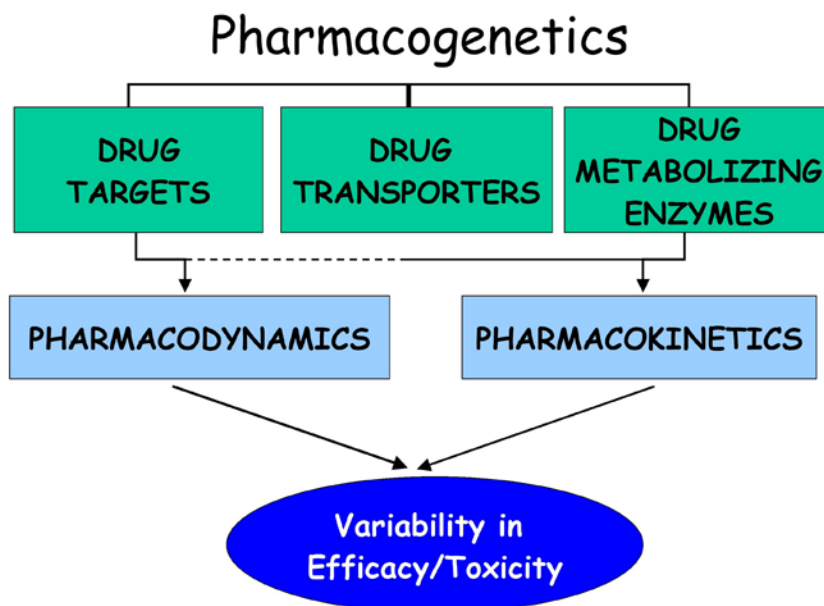
There are a number of studies in the published literature that provide proof-of-concept that genetic variation contributes to the variable response that is observed upon administration of cardiovascular drugs.

In general, knowledge about drugs is separated into two broad categories: pharmacokinetics and pharmacodynamics. Pharmacokinetics describes the disposition of the drug in the body, including absorption from the gut, distribution to tissues, metabolism (usually in the liver), and elimination from the body (usually by the kidneys). Pharmacodynamics involves the pharmacological action of the drug. Similarly, pharmacogenomics studies are also often separated in this way, as highlighted in Fig. 2. Genetic variability in drug-metabolizing enzymes and drug transporters are the most common causes of variable drug pharmacokinetics, and drug targets (defined as any proteins involved directly or indirectly in the pharmacological action of the drug) are the most common source of variable pharmacodynamics.

### *Genetic Influences on Pharmacokinetics*

#### DRUG METABOLISM

Drug-metabolism pharmacogenomics has a long history, with work in this field dating to the 1950s. In many cases, patients with extreme response (often toxicity) were found to have excessively high plasma drug concentrations, which were subsequently found to result from variations in the gene for the enzyme important to that drug's metabolism (4). A number of drug-metabolizing enzymes have inactivating mutations, which lead to absent or nonfunctional protein, and for drugs with a high dependence on that particular enzyme, plasma drug concentrations can be 5–10 times that of normal. For drugs with a



**Fig. 2.** Key components in pharmacogenetics. Figure highlights the two broad areas for research in pharmacogenetics; specifically, pharmacokinetics and pharmacodynamics. Drug-metabolizing enzymes and drug transporters most often contribute to variable pharmacokinetics. Proteins involved in mediating drug effect are broadly defined as drug targets, and include not just the direct protein targets of the drug but any proteins involved in the drug action (e.g., signal transduction proteins, proteins mediating adverse effects). Any of these types of proteins could contribute to interpatient variability in drug efficacy or toxicity, and thus could be candidate genes in pharmacogenetic studies. (Reproduced with permission from ref. 64).

narrow therapeutic index (a small range in which the drug is efficacious, without causing toxicity) these polymorphisms can have profound clinical consequences. One of the best examples of clinical application of pharmacogenomics is with the drug-metabolizing enzyme thiopurine *S*-methyltransferase (TPMT), which metabolizes thiopurines commonly used in cancer chemotherapy and for immune suppression (5). This enzyme has several inactivating mutations, and genetic testing for TPMT prior to administration of thiopurines is becoming the standard of practice (4,5).

In the case of cardiovascular drugs, there are some that are substrates for the enzymes that exhibit genetic variability, but in a number of cases, these do not have important clinical consequences. For example, approx 70–80% of the metabolism of the  $\beta$ -blocker metoprolol is controlled by the polymorphic cytochrome P450 2D6 (CYP2D6) enzyme, and those who have inactivating mutations on both alleles have no functional protein present. In these patients, called poor metabolizers, their metoprolol plasma concentrations can be more than five times that of normal (6–8). And although such patients may require lower doses of metoprolol, when it is dose-titrated to response, poor metabolizers do not appear to experience significantly greater rates of toxicity from metoprolol, in large part because its concentration–response curve plateaus. As such, the marked increases in metoprolol plasma concentration seen in CYP2D6 poor metabolizers do not lead to a greater maximal therapeutic effect, but rather just a prolongation of effect (8).

A number of other  $\beta$ -blockers and antiarrhythmic drugs are metabolized by CYP2D6, including timolol, propranolol, carvedilol, propafenone, flecainide, and mexilitene, although none of these are metabolized by CYP2D6 to as great an extent as metoprolol (9,10). CYP2D6 poor metabolizers will have higher concentrations of these drugs as compared to population averages at any given dose, as is seen with metoprolol. However, it is only on occasion that this translates into important problems clinically.

The examples described above are somewhat typical for the cardiovascular drugs in relation to the clinical impact of genetic polymorphisms in the drug-metabolizing enzymes. In general, cardiovascular drugs have very wide therapeutic indexes, so marked increases in plasma drug concentration are typically well tolerated. Thus, drug metabolism pharmacogenomics is less relevant clinically than in areas where the drugs have a much more narrow therapeutic range, such as is the case with cancer chemotherapy, psychiatric medications, and anticonvulsants.

However, there are some interesting exceptions to this rule. Perhaps the one gathering the greatest attention at present is warfarin, an anticoagulant drug with a very narrow therapeutic range, whose metabolism is governed largely by the polymorphic CYP2C9 enzyme. Unlike CYP2D6, CYP2C9 does not have inactivating mutations, but contains several SNPs that change the encoded amino acid, leading to different functional capacities for metabolism. The most common of these polymorphisms are called CYP2C9\*2 and CYP2C9\*3. Up to 40% of Caucasians (fewer for Asians and those of African descent) carry at least one variant allele, and both variant alleles have significantly reduced catalytic activity (11). Importantly, the presence of one or more variant alleles has been associated in numerous studies with substantially lower warfarin dose requirements, prolongation of the time to stable dosing, and increased risk of bleeding, including serious bleeding episodes (11–14). Given that warfarin requires intensive monitoring and follow-up, the potential benefits of using genetic information to help determine the most appropriate dose for a specific patient are clear. However, studies documenting the benefit of a *priori* genetic information to guide dosing have not yet been done, and until they are, it is unlikely that CYP2C9 genotyping will be used widely in the clinical setting.

Another example in which drug-metabolism genotype is important is with the enzyme *N*-acetyl transferase and the drugs procainamide and hydralazine. The relationship between the genetics and pharmacokinetics of these drugs was among the earliest pharmacogenomic examples. Both of these drugs cause a lupus-like syndrome, and it was recognized decades ago that those who developed this lupus-like syndrome were much more likely to have decreased acetylation as compared with those who did not experience this toxicity (4,15,16). Thus, “slow acetylators,” a genetically-determined phenotype, are at increased risk of drug-induced lupus. In the case of procainamide, fast acetylators may also experience genotype-related adverse events. Specifically, procainamide is metabolized to *N*-acetylprocainamide (NAPA), an active metabolite that also possesses antiarrhythmic activity. Fast acetylators accumulate much higher concentrations of NAPA, and as a result are at somewhat increased risk of NAPA-induced QT prolongation and Torsades de Pointes (TdP).

## DRUG TRANSPORTERS

Another interesting example that relates to pharmacokinetic differences is digoxin and P-glycoprotein. P-glycoprotein is a membrane-bound adenosine triphosphate (ATP)-dependent efflux pump that is important to the distribution and elimination of a number of drugs, and thus falls into the Fig. 2 “transporter” category (17,18). P-glycoprotein vs



gene, *ABCB1* has numerous genetic polymorphisms, some of which have been associated with differences in digoxin plasma concentrations, potentially a result of genotype-dependent bioavailability and renal clearance (19–22). Whereas much of the P-glycoprotein literature for other drugs is inconsistent, the findings with respect to digoxin pharmacokinetics and P-glycoprotein are fairly consistent, a finding that may be attributed to the fact that most other P-glycoprotein substrates are confounded by significant metabolism by CYP3A4, while digoxin is not. Other cardiovascular drugs that are substrates for P-glycoprotein include amiodarone, atorvastatin, diltiazem, losartan, quinidine, and verapamil (17). The impact of P-glycoprotein genetic polymorphisms on their pharmacokinetics remains to be elucidated.

### ***Genetic Influences on Pharmacodynamics***

As highlighted above, there are some interesting examples of cardiovascular drugs whose pharmacokinetics are affected by genetic variation, leading to differences in efficacy or toxicity. However, it seems that genetic variability in the proteins involved in drug pharmacodynamics will ultimately be more informative to understanding response variability for cardiovascular drugs. Unlike drug metabolism pharmacogenomics, for which there are nearly five decades of research, drug target pharmacogenomics (i.e., that focus on drug pharmacodynamics) is in its infancy, with the first cardiovascular pharmacogenomics papers being published in the mid- to late 1990s. Nonetheless, the literature is expanding rapidly, and a comprehensive review is beyond the scope and goals of this chapter. Table 1 provides some examples from the literature, and the reader is referred to several review articles for more detailed information on the cardiovascular pharmacogenomics literature (15,16,23). Herein, we provide selected examples from the cardiovascular pharmacogenomics literature. The information will be divided into studies showing associations between drug efficacy and genotype and those showing associations between drug toxicity and genotype.

#### **VARIABLE DRUG EFFICACY AND PHARMACOGENOMICS**

The two therapeutics areas for which there is the most literature on cardiovascular drug targets and pharmacogenomics are hypertension and lipid disorders. In the area of hypertension, there are data in the literature showing genetic associations between drug targets and antihypertensive response for diuretics,  $\beta$ -blockers, ACE inhibitors, and angiotensin<sub>1</sub>-receptor blockers. The only first-line drug class with no data is the calcium channel blockers, and this is because there are limited data available on sequence variability in genes relevant to the calcium channel blocker response.

In the case of  $\beta$ -blockers, several different studies have shown an association between polymorphisms in the  $\beta_1$ -adrenergic receptor gene and blood pressure lowering (24–26). We and others have found that the strongest predictors of blood pressure lowering are the Arg→Gly polymorphism at codon 389 and the Ser→Gly polymorphism at codon 49. Specifically, those who are Arg389Arg and Ser49Ser had the greatest blood pressure lowering with metoprolol (25). These findings are consistent with in vitro mutagenesis functional studies, which showed that the Arg389 form of the  $\beta_1$ -adrenergic receptor had greater basal and agonist-stimulated adenylyl cyclase activity (27). Similarly, the Ser49 form of the  $\beta_1$ -adrenergic receptor has been shown to be resistant to agonist-stimulated downregulation (28,29). Thus, in vitro data suggested that the Arg389 and Ser49 forms of the receptor would be associated with the greatest sympathetic nervous system activation, and the  $\beta$ -blocker response data support this. One group has also found an asso-

Table 1  
Examples of Cardiovascular Drugs With Evidence  
of Association Between Genetics and Efficacy or Toxicity

<i>Drug/Drug class</i>	<i>Gene(s) associated with efficacy or toxicity<sup>a</sup></i>
$\beta$ -agonists	$\beta_2$ -adrenergic receptors
$\beta$ -blockers	$\beta_1$ -adrenergic receptor <i>ACE</i> Gs protein $\alpha$ subunit <i>CYP2D6</i>
ACE inhibitors	<i>ACE</i> Angiotensinogen <i>AT<sub>1</sub></i> receptor Bradykinin B <sub>2</sub> receptor
Antiarrhythmics	Various congenital long QT syndrome genes <i>N</i> -acetyltransferase (procainamide) <i>CYP2D6</i>
Antithrombotics	
Abciximab	Platelet glycoprotein IIIa
Aspirin	Platelet glycoprotein IIIa
Heparin	Platelet Fc $\gamma$ -receptor
Warfarin	<i>CYP2C9</i>
<i>AT<sub>1</sub></i> -receptor blockers	<i>AT<sub>1</sub></i> receptor
Digoxin	P-glycoprotein
Diuretics	G protein $\beta_3$ subunit $\alpha$ -adducin
Hydralazine	<i>N</i> -acetyltransferase
Lipid-lowering drugs	
Statins	Apolipoprotein E Cholesteryl ester transfer protein Stromelysin-1 $\beta$ -fibrogen LDL receptor Lipoprotein lipase <i>ACE</i>
Gemfibrozil	Apolipoprotein E Stromelysin-1

<sup>a</sup>The reader is referred to focused reviews for detailed information (15,16,23).

ACE, angiotensin-converting enzyme; CYP, cytochrome P450; *AT<sub>1</sub>*, angiotensin type 1; LDL, low-density lipoprotein.

ciation between a G protein  $\alpha_s$  subunit polymorphism and  $\beta$ -blocker response (30). This association is also consistent with pharmacology of the  $\beta$ -blockers, because the  $\beta$ -receptors couple to  $G\alpha_s$ .

For the diuretics, a number of different studies have shown genetic associations between blood pressure response and polymorphisms in the G protein  $\beta_3$  gene and the  $\alpha$ -adducin gene (31–35). In the case of the  $\alpha$ -adducin polymorphism, a nonsynonymous

Arg460Trp cSNP has been associated with increased renal tubular sodium reabsorption, and differential diuretic response (34–36). Perhaps of greatest interest is a study showing that the association between diuretic therapy and reduction in risk of myocardial infarction and stroke differed significantly with  $\alpha$ -adducin genotype (33). This case-control study found that the carriers of the wild-type Gly460Gly genotype had no significant benefit from diuretics, whereas those who carried a variant Trp460 allele had a significant 51% reduction in the risk of nonfatal myocardial infarction and stroke. Although these data need to be confirmed in an independent population, they suggest that genotype information might allow us to target therapies to the patient's underlying hypertension pathophysiology.

Genetic association with responses to cholesterol-lowering therapies, particularly statins have also been extensively investigated (37–47) and summarized (37,47) Genes for which positive associations have been shown include those for apolipoprotein E (ApoE4), stromelysin-1, cholesteryl ester transfer protein (CETP),  $\beta$ -fibrinogen, low-density lipoprotein receptor, lipoprotein lipase, toll-like receptor 4, and CYP3A4, among others. These studies have evaluated a number of different drug-response phenotypes, including lipid lowering, atherosclerosis regression, and cardiovascular outcomes. In the latter category is a study from one of the first statin survival trials, the Scandinavian Simvastatin Survival Study (4S), which showed that carriers of the ApoE4 allele derived the greatest risk reduction from simvastatin therapy (41).

#### CARDIOVASCULAR DRUG TOXICITY AND PHARMACOGENOMICS

Although cardiovascular drugs tend to be safer than drugs used in other clinical situations (e.g., cancer), there are still some important toxicities for which the ability to predict them based on genetic information would be useful. Perhaps highest on the list for potential benefit is the propensity of certain drugs (both cardiovascular and noncardiovascular) to produce prolongation of the QT interval on the electrocardiogram, and increase the risk of the polymorphic ventricular arrhythmia, TdP. QT prolongation and TdP have been the most common reasons for withdrawal of drugs from the US market over the past 10–15 yr, and all new drugs under investigation must undergo extensive testing to determine their ability to produce QT prolongation. As such, there are extensive efforts underway that seek to identify whether there are genetic factors that predispose a patient to developing QT prolongation and TdP. Numerous studies have found mutations (most have allele frequencies  $<0.01$ , thus do not meet the definition of polymorphism) in ion channel genes, particularly the sodium and potassium channels, in patients who have experienced drug-induced QT prolongation or TdP (23,48–52). Although these studies provide clear clues to the genetic basis for risk for drug-induced QT prolongation, there are many challenges. First, QT prolongation and TdP is relatively uncommon, so identifying enough patients who experience this adverse drug effect in order to determine genetic association is a challenge. Additionally, many of the mutations found to date do not occur across a large portion of patients who experience drug-induced QT prolongation. Instead, the data suggest that there are a large number of rare mutations that appear to be associated with QT prolongation risk. Thus, whereas the ultimate goal might be that genetic screening would allow clinicians to select out those patients at risk of QT prolongation from a drug with that propensity, the data are far from that reality. As such, understanding the genetic basis for risk of drug-induced QT prolongation and TdP remains an important research question for basic and clinical scientists.

Another cardiovascular adverse effect that has been extensively studied for its association with genetic polymorphisms is estrogen-induced venous thromboembolism. It is well recognized that estrogen, whether taken as an oral contraceptive or as postmenopausal estrogen replacement therapy, increases a woman's risk of venous thromboembolism. A number of studies have evaluated the role of genetic polymorphisms in proteins in the clotting cascade, particularly prothrombin (Factor II) and Factor V in this adverse event (53). These studies have consistently shown that the G21210A polymorphism in the prothrombin gene and the Leiden mutation (G1691A) in the Factor V gene increase the patient's risk of developing venous thromboembolism. In the case of heterozygotes, the risk is increased approximately three- to eightfold, and in homozygotes about 80-fold (54). The prothrombin and factor polymorphisms occur with allele frequencies in Caucasians of 1–3% and 2–15%, respectively, and thus are common enough in the population to be of interest. Of note is that these mutations are associated with increased risk of venous thromboembolism in the absence of drug therapy, but that risk increases dramatically in the presence of estrogen therapy, particularly through oral contraceptives (53–59). For example, a meta-analysis suggests that the risk of venous thromboembolism is increased 2.3-fold in oral contraceptive users, 5.9-fold in carriers of the Factor V Leiden mutation, and 10.3-fold in carriers of Factor V Leiden who also take oral contraceptives (54). Similar trends have been observed with the prothrombin mutation. As a result of these findings, there has been debate about whether women wishing to use oral contraceptives should first be screened for these mutations (60,61). At present, it is concluded that although the relative risk is markedly increased in individuals with these mutations who also use oral contraceptives, the absolute risk of thromboembolism remains low. Thus, genetic screening at present is not justified, and would likely result in many women being denied oral contraceptives who could take them safely. However, it seems likely that as more factors, both genetic and nongenetic, associated with risk of thromboembolism are identified, such screening might occur in the future.

## DISCOVERY OF GENETIC ASSOCIATIONS WITH DRUG RESPONSE

### *Cardiovascular Pharmacogenomics to Date: A Candidate Gene-Driven Approach*

All of the studies highlighted above can be described as following a candidate gene approach. This means that genes and polymorphisms were selected for study based on suspicion that they might be associated with the disease or drug response of interest. In the case of drug response, this tends to work somewhat better than for diseases because intelligent selection of candidate genes is probably easier. This is because we have prior knowledge of the proteins that are important to either the pharmacokinetics or pharmacological action of the drug. Thus, if we know that a drug is metabolized extensively by CYP2D6, then this becomes a good candidate gene for pharmacogenomic study. Likewise, we can use the information we have about a drug's mechanism of action to help select candidate drug target genes. For example,  $\beta$ -blockers work by inhibiting the actions of norepinephrine at the  $\beta_1$ -adrenergic receptor, thus the  $\beta_1$ -adrenergic receptor gene is a good candidate. Similarly, genetic variability in proteins involved in the signal transduction cascade of the drug response may also contribute much to drug response variability. Candidate genes might also be genes for proteins involved in the system that is interfered with the drug; such is the case for the lipid-lowering drugs. In this case, many

of the candidate genes are ones that are involved in lipid transport and metabolism, not just genes for those proteins directly involved in the drug response. Thus, candidate gene selection in pharmacogenomic research relies on our current knowledge of the drug's pharmacokinetics, mechanism of action, and the physiologic system in which the drug is acting.

#### **PHARMACOGENOMICS KNOWLEDGE PYRAMID USING THE CANDIDATE GENE APPROACH**

Many working in the field of pharmacogenomics view the candidate gene approach as building on a knowledge pyramid, as highlighted in Fig. 3. This is also highlighted in the NIH's Pharmacogenetics and Pharmacogenomics Knowledge Base website (<http://www.pharmgkb.org/>).

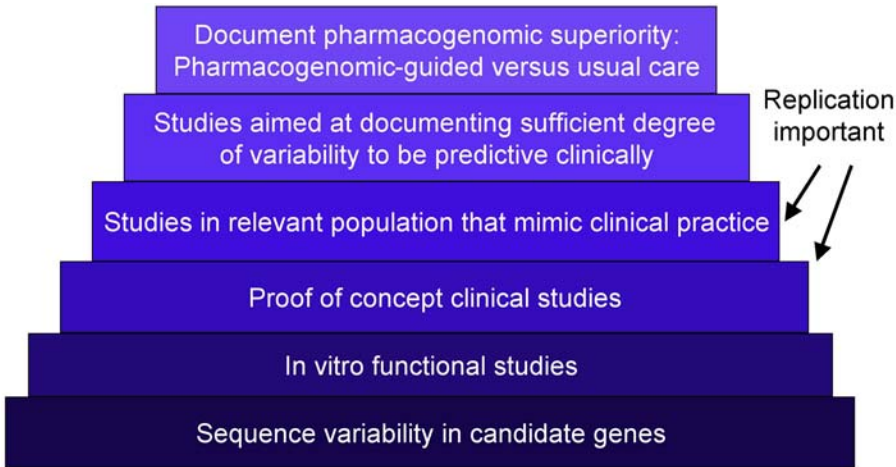
#### **SNP DISCOVERY**

Once a candidate gene is selected, based on the above principles, knowledge of the sequence variability in the gene of interest is at the base of the knowledge pyramid. Although information of polymorphisms is increasingly available in public databases, e.g., <http://www.ncbi.nlm.nih.gov/SNP/>, this information is still often of limited quality, specifically because much of the data currently in the databases has not been validated, or because there is limited information on population allele frequencies. Thus, many pharmacogenomics investigators must first undertake SNP discovery efforts in order to identify the relevant sequence variability. This is often done by resequencing the gene of interest in a sufficient number of individuals (e.g., 40 to 100) of diverse ancestral background (e.g., those of African, European, and Asian descent) to capture the population diversity in the gene.

Most current SNP discovery efforts focus on the coding region of the gene, intron/exon junctions, the 5' untranslated region (which is presumed to contain the promoter), and the 3' untranslated region (where sequence variability may influence mRNA stability). Fewer investigators sequence the entire gene, including the full intronic sequence, because the purpose or function of intronic sequence is not yet clear.

#### **IN VITRO FUNCTIONAL STUDIES**

Once a genetic polymorphism is identified, there is interest in knowing whether it has functional consequences. Some investigators argue that functional studies should be done prior to any clinical association studies, whereas others suggest that functional studies might be reserved until there is evidence of association with a clinical phenotype. For polymorphisms that occur in the coding region and result in a change in amino acid (nonsynonymous cSNPs), there are also approaches that can be taken to predict the likelihood of functional effect. For example, the Grantham score assesses how radical a change the amino acid substitution is, with the assumption that the more radical the change, the more likely it is to have functional consequence (62). Comparisons of sequence homology across species can also provide insight, as amino acid changes that occur in highly conserved regions are more likely to be of functional consequence. Assessment of the location in the protein can also provide insight, because portions of the protein are known to have specific and more important functions than others. For example, in the case of the  $\beta_1$ -adrenergic receptor codon 389 Arg→Gly polymorphism, functional effect was anticipated prior to any studies, for several reasons. First, the nucleotide change resulted in a moderately radical amino acid change, from a charged to uncharged amino acid. Secondly, codon 389 is in a highly conserved region of the  $\beta_1$ -adrenergic



**Fig. 3.** Moving pharmacogenomics to clinical practice. Pyramid of the steps required to move knowledge from point of sequence variability to clinical utility of pharmacogenetic information in practice. (Reproduced with permission from ref. 64).

receptor protein, and is located in a region that is critical to G protein coupling. As described previously, functional studies did suggest that this polymorphism is functional (27), which was further supported by clinical studies, including pharmacogenetic studies (24–26). Finally, if the crystallized protein structure is known, it is possible to make predictions *in silico* of the potential effects of the nonsynonymous SNP.

Actual functional studies can be conducted in a number of ways, although *in vitro* mutagenesis of cell culture systems, with evaluation of the functions of the different forms of the protein, is most common. Transgenic animal models can also be undertaken, but are technically more difficult than the cell culture-based approaches.

Having an understanding of the functional basis for clinical associations with genetic polymorphisms is important, and although some could argue about the point in the process at which these should be conducted, there is little debate about their importance.

### CLINICAL STUDIES

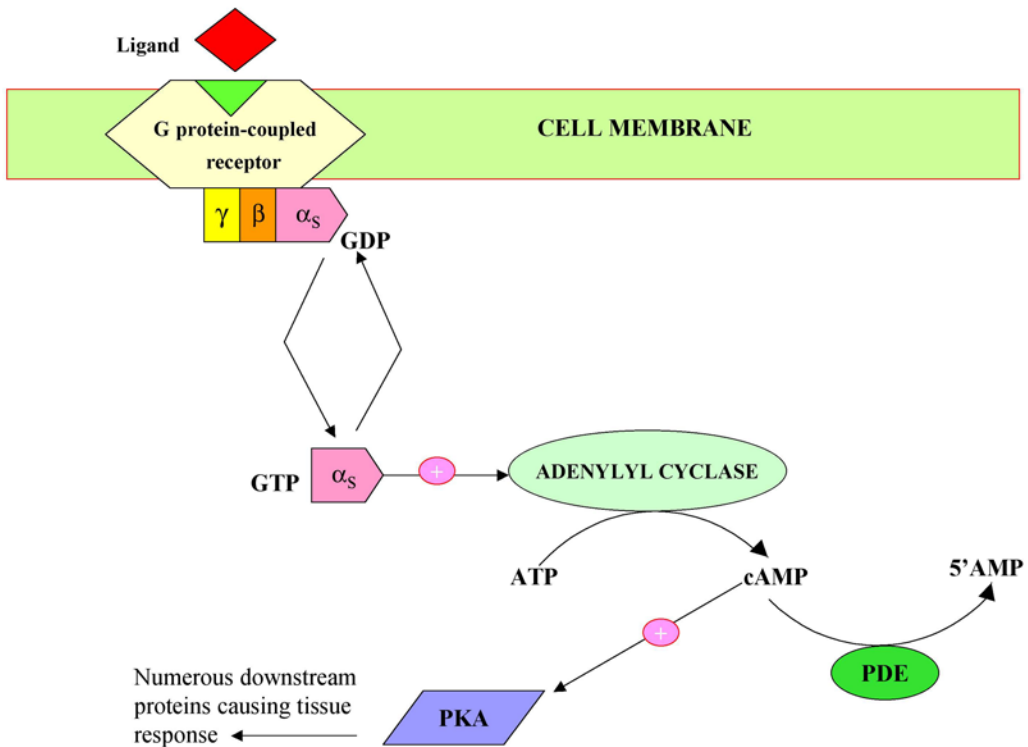
With respect to pharmacogenomics research, clinical studies are sometimes first done in normal volunteers (as compared to the relevant patient population). In certain situations this is reasonable, whereas in others it might be misleading and could lead investigators to believe that there are no genetic associations with drug response, when it is just that the magnitude and variability in drug response in normals may simply not be large enough to document the association.

At some point, particularly for drug target-focused pharmacogenomics studies, it is important that these are conducted in a relevant patient population, in a manner that reflects usual treatment practices. Without this, it will not be possible to move the information to the point of clinical utility.

### *Future Approaches*

#### CANDIDATE GENE APPROACH DRIVEN BY POLYGENIC NATURE OF DRUG RESPONSE

The current literature provides clear proof-of-concept that genetic variability contributes to the interpatient variability in drug response. However, as discussed previously,



**Fig. 4.** Representative signal transduction cascade for drug effect. Simplified schematic of signal transduction cascade for receptors coupling to G protein  $\alpha_s$  subunit. Abbreviations: ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate; PDE, phosphodiesterase; PKA, protein kinase A; GDP, guanosine diphosphate; GTP, guanosine triphosphate. (Reproduced with permission from ref. 64).

most of the studies have focused on a single gene (and often on a single polymorphism within the gene), and although this might provide evidence of a genetic association with response, it usually does not explain enough of the variability to be utilized clinically. This concept is discussed in detail elsewhere (63,64). In order to move the field to the point where a sufficient amount of the response variability is accounted for, it seems likely that there has to be a move toward a more inclusive candidate gene approach or a genomic approach.

A broader candidate gene approach will probably necessitate consideration of genes that contribute to both the drug's pharmacokinetics and pharmacodynamics, and within the pharmacodynamics, consideration of the complexity of the drug response. By example, Fig. 4 shows a simplified schematic of the signal transduction cascade for the  $\beta$ -adrenergic receptors and other  $G\alpha_s$  protein-coupled receptors. This highlights that there are numerous proteins involved in the signal transduction cascade, and thus many genes whose variability might contribute to variable drug response. This signal transduction cascade is not atypical for those involved in drug action, and is less complicated than many of the other signal transduction cascades relevant to drug action. Additionally, there are often other systems that may be involved, and thus their genes may also contribute to variable drug response. For example, the  $\beta$ -adrenergic receptor system is closely tied to the renin–angiotensin system, and thus data suggesting that an *ACE* gene polymorphism is associated with  $\beta$ -blocker response are not surprising (65).

There are several studies that have begun to follow this multicandidate gene approach, and it seems likely that this approach will be increasingly common. For example, Sciarrone et al. (32) examined the *ACE* and  *$\alpha$ -adducin* genes as candidates for response to diuretic therapy in hypertension. These genes were chosen based on evidence for their role in blood pressure response to sodium. Greatest blood pressure response to diuretic therapy was observed in patients with at least one insertion allele of the *ACE* gene and one 460Trp allele of the  *$\alpha$ -adducin* gene, supporting polygenic determinants of diuretic response.

Studies of warfarin pharmacogenetics have also begun to move to this approach. In a study in our laboratory, we are testing the contribution of seven polymorphisms in three coagulation factor genes and the *CYP2C9* gene for their correlation with warfarin dose requirements. Other groups have taken similar approaches. One group focused on genetic variation in three genes that contribute to warfarin pharmacokinetics, including *CYP2C9*, *CYP3A5*, and *ABCB1* (66). Although *ABCB1* variants were overrepresented in the low-dose patients, these polymorphisms were not significant predictors of warfarin dose in multiple regression analysis, whereas *CYP2C9* genotype was. Shikata et al. tested eight different genes, including those for Factors II, VII, IX, and X, protein S, protein C,  $\gamma$ -glutamyl carboxylase and *CYP2C9* relative to warfarin pharmacokinetics and pharmacodynamics (67). They found that they could account for 50% of the variance in warfarin sensitivity based on polymorphisms in *CYP2C9*, Factor II, Factor VII, and  $\gamma$ -glutamyl carboxylase. This level of prediction of variance is higher than nearly all other pharmacogenetic studies to date. These findings illustrate how genetic variation in the proteins involved in both the disposition of a drug and the effects of the drug can contribute to the observed interpatient variability in drug response.

## GENOMIC APPROACHES

Although much has been learned through candidate gene-driven pharmacogenomic studies, there are certain limitations to this approach. One important limitation is that the approach is based on our current understanding of the pharmacokinetics, the mechanism of action of the drug, and the disease state. When comparing pharmacogenomic studies to disease-gene studies, pharmacogenomics clearly has the advantage with regard to the candidate gene approach. This is because we do have a fairly good knowledge of the proteins (and thus genes) involved in the pharmacokinetics and action of the drug, therefore there is greater likelihood of the selected candidate genes being associated with variable drug response. Nonetheless, this approach is still limited by our current understanding, and thus prevents us from identifying genes that might contribute to variable drug response because we do not know of their role in the drug's pharmacokinetics or action.

Another limitation inherent in candidate gene studies is that the genetic variation under study may not actually have any functional activity, despite significant associations between the variant and the outcome of interest. Rather, the variant may be a marker for a functionally active variant or variants located elsewhere in the genome. An alternative to the candidate gene approach is a genomically based approach. One advantage of genome-wide approach is that it requires no previous knowledge of gene function or even of proteins involved in disease biology or drug effects. Genome-wide approaches also have the potential to identify unsuspected genetic associations with drug response, and thus may provide insight into previously undescribed disease mechanisms and targets for disease intervention.



A genomic approach commonly used in animal models and certain human diseases is gene expression microarray studies. This requires collection of a tissue of interest and testing for differential expression levels of mRNA. This approach can be very informative and provide new insights into disease or drug response. However, in the setting of human studies of cardiovascular disease, this approach has clear limitations. First, access to tissues of interest in cardiovascular disease represents a significant barrier to these approaches in human studies. But perhaps more importantly, many of the cardiovascular diseases are essentially systemic diseases, e.g., hypertension, heart failure, or lipid disorders. In these diseases, the most appropriate tissue for study would not be clear. For example, in hypertension, the response to antihypertensive drugs may be based on a combination of their effects on the brain, heart, vasculature and kidney. Thus, gene expression microarray studies have limited utility in cardiovascular pharmacogenomics.

Another approach that is common in the search to understand the genetic basis for disease is the genome scanning approach. This approach tests for association with a phenotype with microsatellite markers spread throughout the genome. This allows for localization on the chromosome where causative genes for the disease of interest may lie. Such approaches are common in cardiovascular disease, and there have been numerous recent publications using such an approach to decipher the genetic contribution to hypertension (68,69). However, this approach is also not feasible within the context of pharmacogenomics studies. This is because these are family-based studies that use large pedigrees or discordant siblings to test for the association. Although family studies are common in genetics research, they are not usually feasible within pharmacogenomics because it is rare that multiple family members, particularly across generations, have exposure to the same drug and have had their response to that drug carefully characterized.

For the reasons previously described, several of the common genomic approaches used to aid in understanding of disease are not particularly amenable to cardiovascular pharmacogenomics research. Genomic approaches that focus on genomic DNA and variability within the genome are therefore the approaches that have the greatest potential application in cardiovascular pharmacogenomics (70,71). To date, published data using such approaches for cardiovascular drugs are limited, although such studies are clearly underway.

Approaches utilizing single nucleotide polymorphism (SNP) maps across the genome are likely to be the most commonly used genomic approaches within cardiovascular pharmacogenomics. The objective under this approach is to use SNPs from across the genome to identify those chromosomal locations associated with drug response variability. SNP maps available commercially at present can test for approx 10,000 SNPs using chip technology (e.g., the Gene Chip® 10K Array from Affymetrix; <http://www.affymetrix.com/products/arrays/specific/10k.affx>) (70). Given that there are an estimated 10 million SNPs, this approach captures only a small portion of the genetic variability, although it is perhaps the best available at this time. A substantial improvement to a genomic SNP map approach occurred in 2004, when Affymetrix marketed a 100,000 SNP chip, based on a similar technology to that of their 10K SNP chip. Eventually, this approach will be perfected further through data generated through the International HapMap Project (<http://www.genome.gov/10001688>). The HapMap project focuses on the fact that the genetic variability occurs in blocks (called haplotypes) within a chromosome (e.g., certain polymorphisms are inherited together at a frequency higher than

expected by chance). The goal of the HapMap project is to determine the SNPs in the haplotype that uniquely define that haplotype, such that only a small portion of the genetic variability in that block would actually have to be tested. It is anticipated that the HapMap project might identify about 500,000 tag SNPs that could be genotyped to represent the genetic diversity across the entire human genome.

The genomic approaches currently available (e.g., the 10K or 100K SNP chips) are expensive, but not prohibitively so. Over the next 5–10 yr, as the costs of genotyping fall further, and the technologies for genome-wide SNP genotyping are enhanced, it is anticipated that costs or feasibility for such approaches will not limit the use of this approach. The greater challenges are likely to lie in the interpretation of the genomic variability data, and the statistical genetics approaches that are needed to analyze these type data.

## LABORATORY METHODS IN PHARMACOGENOMICS RESEARCH: SNP GENOTYPING

The pharmacogenomics researcher will use a variety of laboratory methods, depending on his or her work. Those involved in SNP discovery will utilize direct sequencing, perhaps along with other screening methods for variation such as denaturing high-performance liquid chromatography (HPLC) or single-stranded conformation polymorphism (SSCP) analysis. Those conducting functional studies might use a wide array of cell culture, animal, or molecular techniques in order to test for functional effects of polymorphisms. However, the necessity of being able to genotype samples of genomic DNA from patients is essentially universal among pharmacogenomics researchers.

There are a variety of techniques available for screening variations in an amplified segment of genomic DNA. Direct sequencing of products from a polymerase chain reaction (PCR) is considered the “gold standard” of genotyping techniques to which other techniques are compared. Automated, fluorescence-based DNA sequencing was used to draft the sequence of the human genome and largely enabled the early completion of the human genome project (72). The SNP consortium, a collaborative effort of pharmaceutical companies, a large research charity, and academic centers, used a reduced representation shotgun sequencing approach to create a high-density, high-quality SNP map of the human genome (73). Basically, genomic DNA from a group of unrelated individuals was digested by restriction enzymes, and fragments of certain sizes were then directly sequenced. However, for SNP genotyping (as opposed to SNP discovery), sequencing has several limitations. First, it requires equipment that is typically owned by core laboratories, but much less frequently by individual investigators. This limits the ability of an investigator to do his or her own genotyping if sequencing was the approach. Additionally, sequencing is somewhat more cumbersome from a laboratory and analysis approach than other methods, and in most cases is more expensive than other methods. Thus, it is rarely used as a primary genotyping method.

An alternative to sequencing is PCR coupled with restriction fragment length polymorphism (RFLP) analysis (74). This genotyping approach has been widely used for many years to examine a limited number of polymorphisms in a relatively small number of samples. The PCR-RFLP method involves digestion of PCR products containing the polymorphism of interest with an enzyme that recognizes one polymorphic allele but not the other. Resulting DNA fragments are then separated on a gel by electrophoresis and visualized with ethidium bromide staining to determine genotype. Samples for which the genotype has been determined by direct sequencing may be included in PCR-RFLP

experiments with DNA samples of unknown genotype to ensure the accuracy of genotyping results.

One limitation with RFLP is that restriction enzymes that discriminate between the polymorphic alleles of interest are not always commercially available. However, this limitation may be overcome by creating a restriction site in the PCR product that will then be recognizable by an available restriction enzyme and will enable enzymatic discrimination between polymorphic alleles. Such restriction sites are created through alteration of one or more bases in the oligonucleotide PCR primer. Another limitation of RFLP analysis is that it is not automated, and thus is relatively time-consuming and labor-intensive (and thus expensive), and not practical or feasible for large-scale pharmacogenomic studies.

High throughput genotyping technologies enable efficient genotyping for large-scale pharmacogenomic studies that involve multiple genetic variations and/or genotyping on large sample sets. The available technologies and their advantages, disadvantages, and costs have been reviewed and we will summarize them here (75). Single nucleotide primer extension (SNPE) followed by fluorescence detection is one method to rapidly determine SNPs in a large number of samples (76). The SNPE method is also referred to as minisequencing because a limited number of bases are sequenced with each reaction. Several SNPE assays have been described (77). These include the PinPoint (78) and GOOD (79) assays in which an oligonucleotide primer anneals to a template PCR product immediately adjacent to the polymorphic base pair (bp). In the presence of a fluorescently-labeled dideoxynucleotide triphosphate (ddNTP) chain terminator, DNA polymerase extends the primer by a single base that is complementary to the base on the PCR template. Fluorescence detection with an automated gel-based sequencer reveals the identity of the appended DNA base(s).

In contrast to the PinPoint and Good assays, the PROBE assay (80) and MassExtend™ (81) assay utilize a mixture of deoxynucleotide triphosphates (dNTPs) and a single ddNTP. Deoxynucleotides are incorporated into the primer extension product until the ddNTP is incorporated and the reaction terminates. The resultant primer extension products will differ among heterozygous alleles. Thus the mass of the extension product will differ among alleles. These mass differences can be used to help identify the polymorphism.

Multiplexing, or simultaneous detection of multiple SNPs in a single PCR amplicon, may be done with SNPE and differently-sized primers. Each is designed to end one nucleotide from the polymorphism of interest (82). Separation of differently sized primers on DNA sequencing gels allows for the detection of fluorescently labeled nucleotides appended to each primer. Applied Biosystems (Foster, CA) produces a *SnaPshot*® Multiplex Kit for commercial use, which allows multiplexing of up to 10 SNPE reactions in a single tube ([www.appliedbiosystems.com](http://www.appliedbiosystems.com)).

Mass spectrometry through matrix-assisted laser desorption ionization (MALDI) and time-of-flight detectors is an alternative to fluorescent-labeling as a method to detect appended bases from SNPE assays (83). Genotyping by mass spectrometry is based on the molecular weight differences of nucleotide bases, where the identity of the polymorphic nucleotide is determined by its mass. The combination of SNPE and MALDI-mass spectrometry may be fully automated and is adaptable to high throughput SNP analysis. In addition, multiplexing is possible because of the ability of MALDI-mass spectrometry to accurately resolve primers of various masses (84). Kim et al. (85) recently reported

successful genotyping of three different SNPs in the same PCR amplicon of the  $\beta_2$ -adrenergic receptor gene using SNPE and MALDI-mass spectrometry technology. Several kits are commercially available for SNPE with the MALDI-mass spectrometry genotyping detection platform. These include the Sequazyme™ Pinpoint SNP Assay Kit (Applied Biosystems), and the MassARRAY™ System (Sequenom, San Diego, CA, Caltonics Inc. Billerica, MA, [www.bdal.com](http://www.bdal.com)).

One major limitation with SNPE and MALDI is that they are very sensitive to impurities. Impurities can affect the specificity of nucleotide incorporation by DNA polymerase during the SNPE reaction, in addition to interfering with the mass spectrometry assay (76,77). Thus, purification of the PCR amplicon is necessary prior to the single primer extension, resulting in additional technical time and effort.

Other, non-SNPE methods to produce allele-specific products for mass spectrometry detection include hybridization, oligonucleotide ligation, and cleavage. A detailed review of these technologies in addition to other types of mass spectrometry is provided by Tost and Gut (77). The cost of a mass spectrometer currently limits widespread use of mass spectrometry-based genotyping; however, once the instrumentation is purchased, the cost per sample is comparable with that of other technologies (86).

Pyrosequencing technology is useful for medium- to high-throughput analysis of SNPs and other types of polymorphisms such as insertion/deletions. The methodology of Pyrosequencing is reviewed in detail by Fakhrai-Rad et al. (87). The basic premise of pyrosequencing is that when DNA polymerase incorporates a nucleotide into a DNA strand, pyrophosphate is released. Pyrophosphate is converted to ATP, which provides the necessary energy to generate a detectable light signal that is indicative of the number of incorporated nucleotides. Each of the four dNTPs is added to the pyrosequencing reaction individually. If the base is complementary to the base on a template strand included in the reaction, then the base is incorporated. Unincorporated dNTPs are degraded prior to the addition of the next base. Results from pyrosequencing provide a limited sequence of bases surrounding the polymorphism of interest (usually 20–30 bp). Advantages to pyrosequencing include its accuracy, speed, and automated and multiplexing capabilities. Commercially available models can analyze approx 30,000 polymorphisms in 24 h ([www.biotage.com](http://www.biotage.com)). The sequence surrounding the assayed polymorphism may be used as a control to verify the accuracy of the reaction. The use of pyrosequencing is most limited by the cost of the instrument. However, with the exclusion of instrument cost, the cost of genotyping per sample does not differ significantly from other assay methods. Another limitation is that primers and unincorporated nucleotides from the PCR reaction must be removed prior to pyrosequencing because they may interfere with the pyrosequencing reaction. However, purification strategies amendable to automation are available (87).

Denaturing HPLC is also useful for identifying SNPs, insertions, and deletions (88). Under partial denaturing conditions, PCR products are denatured and reannealed to form homo- and heteroduplexes. Homoduplexes are formed when both DNA strands have the same sequence, whereas heteroduplexes result from the annealing of two strands with differing sequence. Homo- and heteroduplexes are distinguished from one another based on differing retention times. Under complete denaturing conditions, HPLC resolves single-stranded nucleic acids in PCR amplicons based on differences in base composition. Differences in base composition result in differences in hydrophobicity and HPLC retention times. For example, cytosine is the least hydrophobic base and thus has the

shortest retention time, whereas the most hydrophobic base, thymidine, is retained the longest (88). A denaturing HPLC system and software for detecting both known and unknown genetic variations in PCR products, marketed as the Wave<sup>®</sup> System, is commercially available from Transgenomic Inc. (San Jose, CA; [www.transgenomic.com](http://www.transgenomic.com)).

## CONCLUSIONS

The field of pharmacogenomics is a rapidly evolving one, and cardiovascular drugs are among the most extensively studied in the field. There is currently clear evidence that genetic variability influences both the likelihood for efficacy and risk of toxicity of a drug in a given patient. The ultimate goal, from a clinical perspective, is that pharmacogenomics will allow for therapy to be individualized to a specific patient based on his or her genetic profile. This goal is still a long way from reality for cardiovascular drugs; such an approach might be used in limited ways within the 5- to 10-yr time frame, but probably not before that.

The approaches used to address cardiovascular pharmacogenomics questions to date have focused largely on candidate genes. Although a candidate gene approach seems to work better in pharmacogenomics than in studies aimed at uncovering the genetic basis of complex disease, it seems clear that more sophisticated approaches must be taken. These more sophisticated approaches might include either focusing on numerous candidate genes that might influence a drug's action and disposition, or genomic approaches that are not influenced by our current knowledge of the drug.

Technologies to enable pharmacogenomics research are rapidly expanding and the costs for genotyping are dropping quickly. It is anticipated that technologies for genome-wide SNP genotyping will become increasingly affordable, and that the major challenges ahead will center around analysis of the large quantities of data generated by such an approach.

Pharmacogenomics is a field that appears to offer great promise for improving the management of cardiovascular diseases with drug therapy. Research in this field represents a translational approach, with the data collected largely from human subjects, but requiring basic genetic and molecular biology knowledge and techniques in order to conduct the research.

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## Genetic Polymorphisms and Response to HMG-CoA Reductase Inhibitors

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### SUMMARY

Coronary artery disease is among the leading causes of death worldwide. Clinical trials show a protective effect of statins against coronary artery disease. The mean risk reductions for subjects using statins compared with placebo found in these trials is about 30%. These are average reductions for all patients included in the trials. Important factors in interpreting the variability in outcome of drug therapy include the patient's health profile, prognosis, disease severity, quality of drug prescribing, compliance with prescribed pharmacotherapy, and the genetic profile of the patient. This chapter aims to give an overview of the known polymorphisms that have an influence on the effects of statins in the general population. The expectation is that in the future, a subject's genotype may determine whether he/she will be treated with statins or not. Determining the genotype will not deny therapy to a subject, but will help in the decision as to which therapy suits the patient best.

**Key Words:** Pharmacogenetics; HMG-CoA reductase inhibitor; Apolipoprotein  $\epsilon$ ; ACE insertion deletion; CETP; Stromelysin-1;  $\beta$  fibrinogen gene; lipoprotein lipase; hepatic lipase; platelet glycoprotein; toll-like receptor 4; interleukin-1B.

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## INTRODUCTION

Cardiovascular disease is one of the leading causes of death, especially in developed countries. Several risk factors for cardiovascular disease have now been well established, especially cigarette smoking, hypertension, lipid disorders, and diabetes mellitus. Pharmacotherapeutic interventions such as the use of aspirin and other antithrombotic drugs, antihypertensive drugs, and cholesterol-lowering therapy probably have contributed to the gradual decline in cardiovascular disease over the last decades (1,2). Statins are inhibitors of hydroxy-methylglutaryl coenzyme A (HMG-CoA) reductase, which inhibits cholesterol production in the hepatocyte. This increases the synthesis of low-density lipoprotein (LDL) receptors and thereby lowers cholesterol. In recent years, several landmark trials have been published that establish the efficacy of statin therapy in primary and secondary prevention (3–7). Treatment with statins over a 5-yr period was associated with a statistically significant reduction in mortality and in the number of patients experiencing a heart attack or a stroke, or undergoing a revascularization procedure. The risk reduction for coronary artery disease for subjects using statins was approx 30%. These reductions, however, are average effects for all patients included in the trials; in subpopulations these effects may differ.

Important factors in interpreting variability in the outcome of drug therapy include the patient's overall health, prognosis, disease severity, quality of drug prescribing, compliance with prescribed pharmacotherapy, and the genetic profile of the patient (8,9). In the large trials, several subgroups have been analyzed. In the Cholesterol and Recurrent Events (CARE) trial, the effect of pravastatin on the rates of major coronary events was greater among women (–46% [95% CI –22 to –62%]) than among men (–20% [95% CI –8 to –30%]) (3). In the other trials there were no significant differences between men and women. Other subgroup analyses included age groups; comorbidity such as hypertension, diabetes mellitus, previous myocardial infarction, and smoking; and pretreatment plasma lipid levels, but these yielded no statistically significant differences (3–7,10,11). In the Scandinavian Simvastatin Survival study (4S), measurements of the serum cholestanol concentration revealed a subgroup of patients in whom coronary events were not reduced by simvastatin treatment. The reduction in relative risk increased gradually from 0.62 to 1.17 in the quarters of distribution of cholestanol at baseline. Thus, patients with high baseline synthesis of cholesterol seem to be responders whereas those with low synthesis of cholesterol are nonresponders (12).

## PHARMACOGENETICS

Pharmacogenetics and pharmacogenomics are emerging disciplines that focus on genetic determinants of drug response at the levels of single genes or the entire human genome, respectively (13). Polymorphisms may influence drug response in three ways (14). The first way is through pharmacokinetic interactions, caused by genetically based differences in drug absorption, disposition, metabolism and excretion. An important role is played by polymorphisms in the cytochrome P450 (CYP) enzymes. In addition, there are pharmacodynamic gene–drug interactions that involve gene products expressed as receptors or signal transduction molecules, which are relevant to the pharmacodynamics of drugs. After entering the circulation each drug interacts with numerous proteins and multiple types of receptors. These proteins determine the site of action and the pharmacological response. The third possibility of influencing drug response is through genes

that are in the causal pathway of disease and are able to modify the effects of drugs. For example, differences in the effects of statins may be measured on cholesterol levels. However, because statins have certain effects that are independent of cholesterol lowering, such as effects on blood pressure, coagulation, cell proliferation, immune function, and macrophage metabolism (15,16), the effects on cholesterol levels alone may not accurately predict the effects on cardiovascular morbidity and mortality (17). Another way to measure the effects of statins is the rate of progression of coronary atherosclerosis measured by coronary angiography. When a difference in response is measured on these endpoints it is not known whether this drug resistance also translates into a lack of effect on important clinical outcomes (fatal and nonfatal coronary disease).

This chapter aims to give an overview of the currently known polymorphisms that influence the effects of statins. This chapter is an update of a previous publication on this subject (18).

## PHARMACOKINETIC INTERACTIONS

Although several studies have been published recently on possible pharmacokinetic interactions with statins, currently no useful polymorphisms for the prediction of the pharmacokinetics (and thereby predictions of efficacy and adverse effects) are available. Statins are highly extracted by the liver. The CYP enzyme system plays an important part in the metabolism of statins. Most statins (lovastatin, simvastatin, and atorvastatin) are predominantly metabolized by CYP3A4 (19,20). For this enzyme, no functional polymorphisms have been described so far (21).

Simvastatin is given orally as a prodrug and is converted to the active form simvastatin hydroxy acid. Simvastatin is not only metabolized by CYP3A4, but also by CYP2D6 and CYP2C9 (22). Several studies have been published on the role of the CYP2D6 polymorphism in the efficacy of simvastatin. The *in vivo* activity of the CYP2D6 enzyme is characterized by extreme individual variability. The variability in the rate of metabolism by CYP2D6 is more than 100-fold, which is genetically determined (23). To date, 30 polymorphisms in the *CYP2D6* gene have been described, resulting in classification of the phenotype into three categories (24): subjects with normal activity (extensive metabolizers), subjects with low or absent activity (slow metabolizers), and subjects with high activity (ultrarapid metabolizers). It appears that genotyping for the most common defective alleles will predict the CYP2D6 phenotype with a high amount of certainty (24). In 1997, Nordin et al. found in a study of 10 healthy volunteers that subjects who were ultrarapid metabolizers might need higher dosages of simvastatin to be effective (25). Mulder et al. studied 88 patients, with primary and secondary hypercholesterolemia, to investigate the link between polymorphism of CYP2D6 and the efficacy and tolerability of simvastatin treatment. Mulder et al. found results in line with the results of Nordin et al. Adverse effects were more common in slow metabolizers and the efficacy in lowering cholesterol levels was also higher in this group (26). Geisel et al. did not find such an association in a study of 41 patients with primary hypercholesterolemia (27). Several reasons for the different results were discussed. Mulder et al. included patients with secondary hypercholesterolemia and neglected to analyze several nonfunctional alleles and therefore, may have misclassified subjects. On the other hand, the study of Mulder et al. included more patients and therefore, might have had a better chance of finding significant differences (27,28). Because only a few small studies have been performed, larger studies are necessary to assess whether this interaction is relevant.

Polymorphisms in CYP2C9 have been shown to influence response to warfarin (29). Because fluvastatin is also metabolized via this isoform, studies assessing a possible interaction between fluvastatin and CYP2C9 polymorphisms might be of interest (21). Kirchheiner et al. investigated the pharmacokinetics and the cholesterol-lowering activity of fluvastatin in 24 healthy subjects who took 40 mg fluvastatin for 2 wk (30). The pharmacokinetics depended on the CYP2C9 genotype, but differences in plasma concentrations were not reflected in cholesterol (30). Larger studies in hypercholesterolemic patients are needed to confirm these results.

Another pharmacokinetic interaction was published with pravastatin. Pravastatin is not significantly metabolized by the CYP450 system (31). Pravastatin is rapidly absorbed from the upper region of the small intestine, and then taken up efficiently from the circulation by the liver through organic anion transporting polypeptide C (OATP-C), a sodium independent bile transporter (32). A number of single nucleotide polymorphisms have been identified that changed the in vitro transport capability (33). Nishizato et al. investigated the significance of polymorphisms in the *OATP-C* and *OAT3* genes in a Japanese population ( $n = 120$ ) with regard to the disposition kinetics of pravastatin. They provide evidence that the *OATP-C* gene polymorphism contributes to in vivo activity of OATP-C and thereby to differences in the pharmacokinetics of pravastatin. This might lead to differences in the efficacy and safety of pravastatin. Because of the small number of subjects in this study, these results should be confirmed in larger studies (32).

All results in the pharmacokinetic studies discussed in this section are still debated and must be further investigated. Alternative candidate genes for pharmacokinetic interactions include the *CYP3A4* gene, the ABC transporter-like *MDR1* gene, or the glucuronyl transferase gene, because these genes are also involved in the excretion and biotransformation of HMG-CoA reductase inhibitors (21,34).

## GENES IN CAUSAL PATHWAY OF DISEASE

### *Cholesteryl Ester Transfer Protein Polymorphism*

#### REVERSE CHOLESTEROL TRANSPORT

The presence of a polymorphism in the cholesteryl ester transfer protein (*CETP*) gene (which is also called *Taq1B*) is associated with alterations in lipoprotein levels and high-density lipoprotein (HDL) concentrations. The CETP enzyme has a central role in reverse cholesterol transport (RCT), whereby cholesterol from peripheral tissues is transported back to the liver, where it is preferentially excreted into bile. There are several proteins crucial for RCT. These proteins include the ATP-binding cassette transporter 1 gene (*ABC<sub>1</sub>* transporter), lecithin cholesterol acyl transferase (LCAT), CETP, hepatic lipase (HL), and scavenger receptor type BI (SR-BI) (35).

The *ABC<sub>1</sub>* gene product has been provisionally termed cholesterol-efflux regulatory protein (CERP). CERP is involved in the transfer of free cholesterol and phospholipids (PLs) from macrophages to apoA-I. Mutations in the *ABC<sub>1</sub>* gene cause plasma HDL deficiency and premature atherosclerosis (36,37).

Nascent HDL particles, mainly consisting of apo-A1 and PLs, are acceptors for free cholesterol. As soon as these HDL discs have absorbed free cholesterol, the LCAT enzyme converts this cholesterol to cholesterol esters, which increase HDL size from disc to spherical. CETP is then capable of transporting these cholesterol esters to very low-density lipoprotein (VLDL) or LDL. In exchange, CETP converts triglycerides (TGs)

back to HDL. VLDL and, consequently, LDL transport these cholesterol esters back to the liver, where they are taken up through the LDL receptor system. Conversely, in the other pathway, HL hydrolyzes the TGs in HDL (35), which renders HDL a prime candidate for direct uptake into the liver by the SR-BI receptor

### CETP POLYMORPHISM

The presence of the polymorphism in the first intron of the *CETP* gene is referred to as TaqIB. People with the B1 allele have higher CETP concentrations and, because cholesterol esters are extracted from HDL, lower HDL-cholesterol concentrations. CETP levels are highest in subjects with two copies of the B1 allele (B1B1) and lowest in persons with the B2B2 genotype. The mechanism by which the CETP polymorphism may affect CETP activity is not known. It is unlikely that this polymorphism, which is located in an intron, represents a functional mutation. The most plausible explanation is that this polymorphism is in almost complete linkage disequilibrium with a functional polymorphism in the promotor region of the *CETP* gene, the CETP/-629 polymorphism (38,39). This -629 polymorphism, corresponding to a C/A substitution at position -629, exerts a significant effect on transcriptional activity.

Kuivenhoven et al. observed a significant genotype-dependent association of the CETP TaqIB polymorphism with the progression of coronary atherosclerosis in the placebo group; carriers of the B1B1 genotype had the highest CETP concentrations, the lowest HDL-cholesterol (C) concentrations and the fastest progression of coronary atherosclerosis (40). Ordovas et al. found that CETP activity was decreased in men and women with the B2 allele. The HDL concentration for both men and women was lowest in the B1B1 group. For men carrying the B2 allele, the odds ratio for prevalent coronary heart disease was 0.70 (95% CI 0.50–0.98), but after adjustment for HDL-C levels and other known risk factors, the odds ratio was no longer significantly different (0.74 [95% CI 0.46–1.16]). No significant protective effects of this genotype were observed in women (41). There is some controversy in the literature as to whether CETP inhibition is beneficial in preventing ischemic heart disease. Several studies in Japanese populations support the results from Kuivenhoven et al., which showed that CETP deficiency is potentially antiatherogenic and may be associated with an increased life span (42,43). Other studies find an increased risk of ischemic heart disease in subjects with low CETP concentrations even though they have higher HDL-C concentrations (44–48). Van Venrooij et al. found in a 30-wk randomized trial in 217 unrelated diabetic subjects that subjects with the B1B1 genotype or the CC carriers (which were tightly concordant in their study [ $p < 0.001$ ]) had a more atherogenic lipid profile (lower HDL, higher TGs). They also showed that these subjects responded better to atorvastatin with respect to reduction of CETP mass and elevation of HDL-C concentrations (49).

In addition, Kuivenhoven et al. found no difference in plasma lipoprotein response to pravastatin between the different genotypes, but the B2B2 genotype (about 16% of all Caucasian men) with low CETP and high HDL concentrations did not respond to pravastatin in terms of decreased disease progression (40). Furthermore, in a case-control study, Fumeron et al. showed that HDL-C levels were only increased in subjects with the B2B2 genotype after ingesting at least 25 g of alcohol per day (50). In the cohort of Kuivenhoven et al., alcohol consumption did not affect the association between the CETP genotype and the angiographic outcome in the control group, nor in the group treated with pravastatin (51). In the WOSCOPS trial (one of the primary prevention trials) the same

association between the B2B2 genotype and cardiovascular events was found. Homozygotes for the B2B2 allele had a higher HDL-C and a 30% reduction of suffering a cardiovascular event compared to B1B1 homozygotes. This association was primarily found in nonsmokers, not in smokers. However, no interaction was observed between the *CETP* genotype and pravastatin treatment (52). They also typed the -631 C/A, -629 C/A, I405V, and D442G *CETP* polymorphisms but they did not find significant association with risk. Haplotype analysis did not add to the information given by the individual polymorphisms (52). Blankenberg et al. found in the Atherogene study, a cohort study including 1303 patients who had at least one stenosis greater than 30% in a major coronary artery diagnosed with coronary angiography, that in patients with coronary artery disease, the *CETP* -629 A allele (which is almost completely concordant with the B2 allele of the Taq IB polymorphism) had a strong protective effect on future mortality from cardiovascular causes. Furthermore, a statistically significant interaction between the *CETP* -629 C/A polymorphism and statin treatment on cardiovascular mortality was found. The benefit of statin treatment was restricted to subjects with the -629 C allele. In these patients, cardiovascular mortality was reduced by about 50% in those taking statin medication, whereas no effect was observed in patients carrying the -629 A allele (53).

### ***Stromelysin-1 Polymorphism***

Matrix metalloproteinases (such as stromelysin) have important roles in connective tissue remodeling during tissue repair, cell migration, angiogenesis, tissue morphogenesis, and growth (54–57). These physiological processes require a tightly controlled balance between matrix metalloproteinases and their specific tissue inhibitors. Disruption of this balance may lead to various pathological states. Low tissue levels of these metalloproteinases are associated with atherosclerosis (54). A common polymorphism in the promoter sequence of the stromelysin-1 gene exists, with one allele having a run of six adenosines (6A) and the other of five adenosines (5A). The prevalence of the two alleles 5A and 6A was found to be 51% and 49% in a sample of 354 healthy individuals in the United Kingdom (54). This promoter variant affects transcription. In vitro studies have shown that genotypes with a 5A allele expressed higher activity of stromelysin-1 than the 6A allele in both cultured fibroblasts and vascular smooth muscle cells (54). Compared with other genotypes, individuals homozygous for the 6A allele would have lower stromelysin-1 levels in their arterial walls because of lower transcription level, and this might therefore favor deposition of extracellular matrix in the atherosclerotic lesions. This may lead to the development of an atherosclerotic plaque with a thick cap, and result in a more rapid progression of angiographically defined stenosis (58).

In the REGRESS trial of pravastatin vs placebo, de Maat et al. compared male patients with coronary artery disease with 5A5A, 5A6A, and 6A6A genotypes of the stromelysin polymorphism, with respect to relevant clinical events such as myocardial infarction (fatal or nonfatal), mortality of coronary heart disease, percutaneous transluminal coronary angioplasty or coronary artery bypass graft surgery, stroke and transient ischemic attack, and overall mortality (59). Patients in the placebo group with the 5A6A or 6A6A genotypes had a higher 2-yr cumulative incidence of one of these clinical events than patients with the 5A5A genotype (26% and 12%, respectively;  $p = 0.03$ ). In the pravastatin group, the risk of clinical events in patients with 5A6A or 6A6A genotypes was lower (14%) than in patients on placebo, whereas it was unchanged in those with a 5A5A genotype (17%) (see Table 1). These effects were independent of the effects of pravastatin on lipid levels (59).

Table 1  
Polymorphisms That Might Influence the Efficacy of Statin Therapy

<i>Polymorphism</i>	<i>Effect</i>	<i>References</i>
Cytochrome P450 2D6	Slow metabolizers may have higher efficacy and more side effects of simvastatin	25–28
Cytochrome P450 2C9	No effect found with fluvastatin	30
OATP-C	Disposition kinetics of pravastatin were different.	32
CETP Taq IB and –629 C/A	Subjects with the B2B2 or with –629 AA allele had no effect from statin therapy on reduction of angiographically defined CHD and on reduction of cardiovascular events.	40,51–53
Stromelysin-1	Subjects with the 5A5A genotype had no effects of pravastatin on incidence of clinical events.	59
$\beta$ -fibrinogene –455 G/A and TaqI	Subjects with the –455 AA genotype had a greater efficacy of statins.	67
Apolipoprotein E	Subjects with the $\epsilon$ 4 allele have the least cholesterol lowering effect with statins, but they have the same or even a better effect of statins (compared with the $\epsilon$ 2 and $\epsilon$ 3 allele) in reducing CHD.	72,74–85
Lipoprotein lipase gene	Subjects with the Asp9Asn mutation had a greater efficacy of statins on angiographic parameters.	91
ACE insertion deletion	Results for this polymorphism are conflicting.	78,100–103
Hepatic lipase –514 C/T	Subjects with the TT genotype did not have a regression of angiographically determined atherosclerosis after 2.5 y of intensive lipid-lowering therapy.	108
Platelet Glycoprotein	Statin therapy reduced the increased rate of restenosis associated with the P1 <sup>A2</sup> allele.	100,110
Toll-like receptor 4	Carriers of the 229 Gly had a much stronger cardiovascular risk reduction, but the interaction was not found in reduction of lipid parameters.	117
Interleukin-1B	Coronary function improves after 6 mo of statin therapy in subjects with the A2- allele but not in subjects with the A2+ allele.	118

OATP, organic anion transporting polypeptide; CETP, cholesteryl ester transfer protein; ACE, angiotensin-converting enzyme; CHD, coronary heart disease.

### –455G/A AND TAQ I POLYMORPHISMS OF THE $\beta$ -FIBRINOGEN GENE

Several epidemiological studies have reported a strong association between elevated plasma levels of fibrinogen and an increased risk of myocardial infarction (60–65) and stroke (66). Because fibrinogen is an acute phase protein, an increased plasma fibrinogen level may reflect the inflammatory state of the vascular wall and may thus be related to cardiovascular risk (64). The three chains of fibrinogen are encoded by three different genes (68). Polymorphisms of the  $\alpha$  gene (*Taq I*) and of the  $\beta$  gene (–455G/A) are associated with differences in plasma levels of fibrinogen.

De Maat et al. found in The REGRESS trial significantly higher fibrinogen levels (3.9 g/L [95% CI 3.2–4.8]) in subjects who were homozygous for the rare –455A allele (the



frequency was 0.21%), whereas the homozygotes for the common -455G allele and the heterozygotes had comparable fibrinogen levels (3.2 g/L [95% CI 3.0–3.3], and 3.1 g/L [95% CI 2.9–3.3], respectively) (67). In the placebo group, subjects with the -455AA had a significantly greater progression of coronary artery disease, as reflected by angiographic variables (mean segment diameter decrease  $0.24 \pm 0.45$ ), than patients with the -455GG and the -455GA genotypes (mean segment diameter decrease  $0.09 \pm 0.39$  and  $0.10 \pm 0.39$ , respectively). In patients receiving pravastatin, this difference was not observed. These results suggest that pravastatin is capable of offsetting the deleterious effects of the -455AA genotype of the  $\beta$ -fibrinogen gene (67).

#### APOLIPOPROTEIN- $\epsilon$ 4

Several studies have investigated the relation between apolipoprotein- $\epsilon$  (apo $\epsilon$ ) genotypes and the LDL cholesterol response to HMG-CoA reductase inhibitors in hypercholesterolemic patients. Human apo $\epsilon$  is a genetically polymorphic protein defined by three alleles— $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4—at a single gene locus on chromosome 19; these code for three isoforms ( $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4) that differ by an amino acid substitution at residues 112 and 158, and thus determine the six genotypes resulting from the combination of any two of them (69). Apo $\epsilon$  allele frequencies in the normal population were assessed in 2457 subjects in the Framingham Offspring study and were 8%, 78%, and 14% (70), respectively, for  $\epsilon$ 2,  $\epsilon$ 3, and  $\epsilon$ 4; Corbo et al. investigated the worldwide distribution of the apo $\epsilon$  polymorphism and discovered that the  $\epsilon$ 3 allele is the most frequent in all human populations and that its frequency is always negatively correlated with that of  $\epsilon$ 4 (71). The  $\epsilon$ 4 allele occurs relatively frequently in human populations where foraging still exists or food supplies are scarce or qualitatively poor. Carrying this allele would be favorable, because this allele promotes intestinal cholesterol uptake and increases plasma cholesterol levels which otherwise would be too low (71).

The polymorphism of the apo $\epsilon$  genotype influences hepatic cholesterol content because lipoproteins with the  $\epsilon$ 4 isoform are taken up with greater affinity than those with the common  $\epsilon$ 3 isoform, which in turn are cleared more efficiently than those with the  $\epsilon$ 2 isoform. Accelerated lipoprotein clearance by the liver leads to a downregulation of hepatocyte LDL receptors. This, in addition to increased intestinal uptake, underlies the well-known hypercholesterolemic effect of the  $\epsilon$ 4 allele, with its attendant high risk of atherosclerosis and cardiovascular mortality (72). On the other hand, lipoproteins containing apo $\epsilon$ 2 have a reduced binding affinity for the LDL receptor; thus their plasma clearance rate is reduced. This lowers intracellular cholesterol levels and upregulates HMG-CoA reductase synthesis. HMG-CoA reductase inhibitors (statins) may be less effective in reducing cholesterol levels in apo $\epsilon$ 4 individuals, as they may already have low HMG-CoA reductase activities (44,73). Several studies found a significant interaction between an individual's apo $\epsilon$  genotype and plasma lipoprotein-lipid changes with statin therapy (74–78). In these studies, subjects with the  $\epsilon$ 2 allele, and sometimes subjects with the  $\epsilon$ 3 allele, were more likely to respond favorably to statin therapy than subjects with at least one  $\epsilon$ 4 allele. The decrease in both total cholesterol and LDL cholesterol was larger in subjects with the  $\epsilon$ 2 allele than in subjects with an  $\epsilon$ 3 or an  $\epsilon$ 4 allele. In subjects with the  $\epsilon$ 3 allele, total cholesterol and LDL cholesterol were reduced more than in subjects with at least one  $\epsilon$ 4 allele. Five other studies did not find significant differences in the cholesterol lowering effects of statins in the different apo $\epsilon$  genotypes (72,79–82). However, in two of these studies there was a trend for greater LDL cholesterol reductions in subjects with the  $\epsilon$ 2 and  $\epsilon$ 3 allele (80,82). Two studies reported that,

whereas men displayed a significant gene–drug response interaction, in women the apoE polymorphism did not account for any significant gene–drug interaction (76,83). It was also found in the REGRESS study that  $\epsilon 2$  carriers exhibited the largest improvement in lipoprotein levels upon pravastatin treatment, compared with those having  $\epsilon 3$  or  $\epsilon 4$  alleles, but the efficacy of pravastatin toward angiographic parameters was less pronounced in carriers of the  $\epsilon 2$  allele, although this was not statistically significant (84). Importantly, in a substudy from the 4S study myocardial infarction survivors with the  $\epsilon 4$  allele proved to have a nearly twofold increased risk of dying in a follow-up period of about 5.5 yr, compared with other patients. Compared with placebo, the relative risk of mortality in subjects treated with simvastatin was 0.33 (95% CI 0.16–0.69) in  $\epsilon 4$  carriers and 0.66 (95% CI 0.35–1.24) in other patients (*see* Table 1). The increased risk of death was not accompanied by an increased risk of a major coronary event, and there was also no difference in the efficacy of statin treatment on reducing coronary events (81).

In a hypercholesterolemic subcohort ( $n = 3626$ ) of the Rotterdam study, a large cohort study that included 7983 subjects of 55 yr and older in a suburb of Rotterdam, no differences in effect were found between subjects with and without the  $\epsilon 4$  allele in reducing the risk of coronary artery disease and total mortality. The mortality risk in subjects with the  $\epsilon 4$  allele was reduced to 0.71 (85) after 2 yr of statin treatment, whereas the mortality risk in subjects without the  $\epsilon 4$  allele was reduced to 0.91 (85). The difference with the 4S study might be explained by the difference in mean age of both populations. Subjects in the Rotterdam study had a higher mean age and, unlike the subjects in the 4S study, in the untreated subjects there was no increased risk of dying in subjects with the  $\epsilon 4$  allele. Perhaps risk factors other than carrying the  $\epsilon 4$  allele became more important at a higher age in predicting the risk of dying (85). Furthermore, among subjects in the Rotterdam study that started statin therapy, the risk of discontinuing medication within 3 yr was 3.18 times higher in men with the  $\epsilon 4\epsilon 4$  genotype compared to men with the  $\epsilon 2\epsilon 3$  genotype (86). This suggests that subjects who are genetically prone to develop hypercholesterolemia showed the highest risk of discontinuation of statin treatment. A possible explanation for this observation is that these men have the least effect on their cholesterol levels. This may lead to the conclusion that measuring cholesterol levels might not be a good way to evaluate the efficacy of statin therapy (86).

#### MUTATIONS IN THE LIPOPROTEIN LIPASE GENE

Lipoprotein lipase is a multifunctional protein and pivotal enzyme in lipoprotein metabolism. It is anchored to the vascular endothelium where it constitutes the rate-limiting step in the catabolism of TGs in circulating TG-rich lipoproteins (87,88). The contribution of lipoprotein lipase to atherogenesis is significantly influenced by the balance between vessel wall protein (proatherogenic) and plasma activity (antiatherogenic) (89).

Maily et al. found in their study that the frequency of healthy Dutch, Swedish, English, and Scottish carriers of the Asp<sub>9</sub>Asn mutation (Asp substituted by Asn at position 9 in exon 2) was 1.6 to 4.4% (90). The frequency of the carriers was roughly twice as high (range, 4.0–9.8%) in selected subjects with combined hyperlipidemia (elevated plasma levels of cholesterol and TGs) and in patients with angiographically assessed atherosclerosis (REGRESS) (91). Carriers of the mutation more often had a family history of cardiovascular disease and higher TG and lower HDL-C levels than noncarriers.

The reduction of total and LDL cholesterol appeared to be less in patients with the mutation compared to patients without the mutation. However, the differences between

patients with and without the mutation did not differ significantly between the placebo and the pravastatin groups (91). Angiographic results (progression of focal atherosclerosis and percentage diameter stenosis) showed a deleterious effect of the mutation which could be reversed by pravastatin therapy (91). The relative risk of the presence of the Asp9Asn mutation for any clinical event within 2 yr was estimated to be 1.85 (95% CI 0.94–3.66). The effect of pravastatin on the clinical event-free period was not significantly different for patients with and without the mutation (91).

Although the lipid-lowering effect was attenuated in patients carrying the Asp<sub>9</sub>Asn mutation, the deleterious effect of this mutant on the progression of atherosclerosis could be reversed by pravastatin.

Two other common polymorphisms in the lipoprotein lipase gene are associated with coronary heart disease. The Ser447X polymorphism is seen in 17–22% of the population. This polymorphism was found to be protective against high TG levels, low HDL-C cholesterol levels, and premature coronary heart disease (92–94). The N291S is seen in 1–7% of the population. This polymorphism is associated with elevated TGs, decreased HDL cholesterol, and, most likely, increased heart disease risk (93,94). It is not yet established if the effects of statins differ in subjects with these polymorphisms.

#### ACE DELETION-TYPE GENE

The angiotensin-converting enzyme (ACE) is thought to play an important role in the development of coronary artery disease. In humans, plasma levels of ACE are partly under genetic control. Plasma and cellular levels of ACE are associated with the insertion/deletion (I/D) polymorphism located in intron 16 of the ACE gene. DD carriers have about twice the plasma levels of ACE compared with II carriers, whereas heterozygotes have intermediate levels (95). The frequencies of the ACE genotypes differ in various populations. In the control group of the male Caucasian study population of Gardemann et al., the frequencies of II, ID, and DD were 23, 50, and 27%, respectively (96). The angiotensin converting enzyme converts angiotensin I into the bioactive angiotensin II, and is also responsible for the breakdown of bradykinin to kinin degradation products. These processes result in an increased vascular tone, neointimal proliferation, and LDL oxidation, all predisposing to atherosclerosis (97). Many studies have examined the correlation of the different genotypes with various cardiovascular diseases. Cambien et al. were the first to report an association of this deletion polymorphism with an increased risk of coronary artery disease (98). The meta-analysis of Staessen et al. included 145 reports with an overall sample size of 49,959 subjects (99). In comparison with the II reference group, the excess risk in DD homozygotes was 32% for coronary heart disease (CHD; 30 studies) and 45% for myocardial infarction (20 studies). The D allele behaves as a marker of atherosclerotic cardiovascular complications (99). Concerning the influence of the ACE genotype on the effectiveness of statins, however, contradictory results have been published.

Marquez-Vidal et al. found no interaction of the ACE polymorphism and statins on reduction of lipids and lipoproteins in their case-control study (78). In the CARE trial, subjects with the Platelet Pla2 allele ACE D allele carriers had a larger risk reduction than ACE II subjects (100). In the Lipoprotein and Coronary Atherosclerosis study (LCAS) study, subjects with the ACE DD genotype had the strongest reduction of coronary atherosclerosis with pravastatin. The distribution of clinical events among the genotypes

was not clinically significantly different (101). In The REGRESS trial comparing pravastatin with placebo, van Geel et al. found that the DD genotype was associated with a significantly higher incidence of ischemic events. Although pravastatin decreased serum lipids to a similar extent in the DD as in the ID and II groups, the beneficial effects of pravastatin on angiographically defined coronary atherosclerosis were apparently blunted by the ACE deletion type DD gene (102). Treating patients with the DD genotype with pravastatin seems less effective than treating patients with the other genotypes with pravastatin.

In a hypercholesterolemic cohort in the Rotterdam study ( $n = 3624$ ), an association between the ACE genotype and the effectiveness of statins in reducing coronary events was found. The relative risk reduction in DD subjects was 1.29 (95% CI 0.62–2.60), in ID subjects, 0.87 (95% CI 0.52–1.45), and in II subjects, 0.40 (95% CI 0.15–1.10). In men, this interaction was much stronger than in women (103). In men the relative risk reduction in DD subjects was 1.34 (0.44–4.09), and in II subjects 0.23 (0.04–1.28); in women, the relative risk reduction in DD subjects was 1.27 (0.43–3.81), and in II subjects, 0.72 (0.20–2.56). The gender difference in risk reduction is not easily explained. Other studies showed that genetic variance in the region of the ACE gene significantly influenced interindividual blood pressure in males, but not in females (104, 105). This suggests that there are gender differences in genetic regulation of the ACE system. There might be an interaction between the ACE genotype and statin therapy in men, but these data need to be confirmed in a larger study.

#### –514 CT POLYMORPHISM IN HL GENE

HL is a plasma lipolytic enzyme that plays a major role in the metabolism of LDL cholesterol and HDL cholesterol. HL promotes the conversion of large TG-rich HDL2 to small, dense HDL3 particles. High HL levels are associated with low HDL2 levels. In addition, HL catalyzes the hydrolysis of TGs and PLs of the intermediate density lipoproteins (IDL) and large buoyant LDL to form the more atherogenic small, dense LDL particles (106). Patients with small, dense LDL have an increased risk of coronary artery disease (107). The presence of a C instead of a T at position –514 with respect to the transcription start site of the HL gene accounts for 20–30% of the variance in HL activity in men and women (108). The presence of the C allele contributes to increased HL activity, which leads to more atherogenic LDL particles and lower levels of anti-atherogenic HDL cholesterol.

Zambon et al. conducted a study within a small but intense clinical trial (108). They studied 49 dyslipidemic men who were treated for 2.5 yr with intensive lipid-lowering therapy with either lovastatin and colestipol, or niacin and colestipol. The type of lipid-lowering therapy did not affect the association between polymorphism and changes in coronary stenosis. The results from the two different treatment groups were therefore pooled and analyzed together. Homozygous CC patients showed a greater decrease in HL activity and a greater increase in LDL buoyancy with lipid-lowering therapy than subjects with at least one T allele.

Lipid-lowering therapy resulted in a significant improvement of coronary stenosis in CC patients ( $\Delta\%_{\text{Sprox}} -2.1$ ) and to a lesser extent in the TC group ( $\Delta\%_{\text{Sprox}} -1.1$ ), whereas progression of stenosis ( $\Delta\%_{\text{Sprox}} 4.0$ ) was observed in the TT group. Because no distinction was made between the two lipid-lowering therapies, it is not clear if the differences in treatment effect were attributable to statin therapy.

## PLATELET GLYCOPROTEIN

Platelets play a central role in the restenosis process by inducing neointimal proliferation after coronary interventions (109). The glycoprotein IIb/IIIa  $PI^{A2}$  polymorphism has been associated with the occurrence of acute coronary syndromes and increased restenosis rates. Statins have been shown to exert potent antiproliferative, anti-inflammatory, and antithrombotic properties, thereby potentially interfering with the major processes of in-stent restenosis. To investigate if statin therapy affects restenosis rates Walter et al. followed 650 patients for 6 mo after coronary stent insertion. Carriers of the  $PI^{A2}$  allele (22% of the patients) demonstrated a significantly increased restenosis rate, which was diminished by statin therapy (50.9% vs 28.6%,  $p = 0.01$ ). Patients homozygous for the  $PI^{A1}$  allele only had a slight reduction in stenosis rate (27% vs 34%,  $p = 0.13$ ). Moreover, statin therapy was associated with a significant reduction (28.2% vs 49.3%) of myocardial infarction, cardiac death, and target vessel revascularization in the 6 mo after the intervention in patients with the  $PI^{A2}$  allele, and only with a minimal reduction in subjects with the  $PI^{A1}$  allele (32.1% in statin treated patients and 37.5% in patients without treatment). Statin therapy reduces increased stent restenosis rates and improves clinical outcome following coronary stent implantation in patients bearing the  $PI^{A2}$  allele, suggesting that statins interfere with the functional consequence of a genetically determined platelet-mediated risk factor associated with  $PI^{A2}$  polymorphism (110).

The clinical effect of the  $PI^A$  genotype has also been investigated in a substudy of the CARE trial (100). The prevalence of the  $PI^{A1.A2}$  genotype was 28.8% in the cases and 23.8% in controls; this was not significantly different. The relative risk of CHD death or nonfatal myocardial infarction in the  $PI^{A1.A2}$  genotype group was 1.32 (95% CI 0.99–1.76). This suggests that there is a trend towards a higher relative risk, but it is not statistically significant. In patients with the  $PI^{A1.A2}$  genotype, treatment with pravastatin reduced the risk of fatal CHD or nonfatal myocardial infarction by 31%, whereas in patients with the  $PI^{A1.A1}$  genotype, treatment with pravastatin only reduced these events with 7%. Of the seven patients with the  $PI^{A2.A2}$  genotype in the control group only one experienced a fatal CHD or a myocardial infarction, whereas in the pravastatin group six of the nine  $PI^{A2.A2}$  patients experienced such an event. These data suggest that the  $PI^{A2.A2}$  genotype is not associated with an increased risk of death resulting from CHD or nonfatal myocardial infarction, and that pravastatin might have a detrimental effect on coronary events in patients with this genotype (100,111). Because of the low sample size in this study, these results should be cautiously interpreted.

## TOLL-LIKE RECEPTOR 4

Atherosclerosis is increasingly considered to be a chronic inflammatory disease (112). Lipopolysaccharide (LPS) is a product of Gram-negative microorganisms that activates the immune system. LPS in combination with CD14 serves as a ligand to toll-like receptor 4 (TLR4). The presence of LPS in the circulation is not confined to sepsis, but also occurs in healthy subjects (113,114) and is associated with early atherosclerosis (113). Two functional SNPs have been discovered in the *TLR4* gene. These variants, Asp299Gly and Thr399Ile, lead to a blunted immunological response to inhaled LPS (115) and to lower levels of proinflammatory cytokines, acute-phase reactants, and soluble adhesion molecules (116). Most importantly, they were associated with reduced extent and progression of angiographically-determined carotid atherosclerosis (116). Boekholdt et al. investigated in the REGRESS study whether the Asp299Gly and the Thr399Ile polymor-

phisms influenced the progression of coronary atherosclerosis and the risk of cardiovascular events (117). There were no significant differences between genetically defined subgroups with respect to baseline factors, treatment, or in-trial changes of lipid, lipoprotein, or angiographic measurements. Carriership of the 299Gly allele did not significantly affect the risk of cardiovascular events in the entire cohort when compared with noncarriers (11.5% vs 14.9%,  $p = 0.58$ ). However, in the pravastatin group, carriers of the 299Gly allele had a significantly lower risk of cardiovascular events than noncarriers. Among noncarriers, the risk of cardiovascular events was reduced from 18.1% to 11.5%, whereas among carriers of the 229Gly the risk was reduced from 29.6% to 2%. Testing for the interaction between the 299Gly genotype and pravastatin was statistically significant ( $p = 0.025$ ) (117). Analyses were performed to investigate whether the Thr399Ile polymorphism influenced the interaction between 299Gly and pravastatin, but the results did not allow any conclusions because of the low frequency of this genotype (117). This is another observation of an interaction between a genotype and statin treatment in reducing clinical events without an interaction reducing lipid parameters.

### INTERLEUKIN-1B GENOTYPE

A polymorphism at position -511 of interleukin (IL)-1B gene promoter regulates IL-1B levels, immune and inflammatory responses, and possible atherogenesis. Lehtimäki et al. found in a small trial ( $n = 34$ ) that at baseline, there was no difference in basal or adenosine-stimulated myocardial flow between subjects with the A2+ and subjects with the A2- genotype. After 6 mo of treatment with pravastatin, the adenosine-stimulated myocardial flow increased by 18.0% in subjects with the A2- genotype ( $n = 7$ ), and decreased by 2% in subjects with the A2+ genotype ( $n = 7$ ). In the placebo recipients there were no significant changes compared with the baseline values. Both genotype groups showed a similar decrease in total LDL cholesterol levels. Coronary function improves after 6 mo of statin therapy in subjects with the IL-1B A2- allele but not in those with the A2+ allele (118).

## CONCLUSIONS

In this review, we have discussed a number of polymorphisms which might interfere with the effectiveness of statin therapy. Most polymorphisms described in this chapter have an indirect effect on statin response. Only a few of them are in genes encoding for proteins that are involved in the disposition of statins, or in genes-encoding proteins that are direct targets of statin therapy. More proteins are involved in efficacy and metabolism of statins; therefore, more genes are involved, and probably more polymorphisms will influence the efficacy of statin therapy. The use of other techniques in pharmacogenetic and pharmacogenomic research will enable us to compare gene profiles of patients with differences in reactions to drugs. Instead of looking at one single SNP in a gene, haplotypes might better explain the differences in response to drugs. Furthermore, models need to be developed that enable us to look at combinations of polymorphisms at the same time. Because so many genes are involved, probably more polymorphisms have to be determined to predict a patients response to statins.

What is the clinical relevance of the polymorphisms described in this chapter? Some of them might be useful to select patients with a high risk for coronary artery disease (for example, ACE and ApoE). Subjects with that particular genotype might be treated rigorously, especially in the case of Apoε4 carriers, who are genetically prone to develop coronary artery disease and who are at higher risk to discontinue their therapy (86). Other

polymorphisms could predict the effectiveness of statins. For example, carriers of the CETP B2B2 polymorphism or the stromelysin-1 A5A5 polymorphism seem to have no benefit from treatment with statins. Nevertheless, in the study of Kuivenhoven et al., it was only the effect on angiographic progression of coronary disease that was measured, whereas statins do have effects that are independent of cholesterol lowering. Therefore, we do agree with Goldstein (119) that at this moment, there is certainly not enough evidence to exclude patients with a certain genotype from statin treatment. However, in the near future, it is likely that genetic information may be used to modify dose and suggest supplemental treatment regimens. Further research in large general population samples and large clinical trials is necessary to assess the importance of these polymorphisms on the effectiveness of statins in the reduction of cardiovascular diseases.

The ethical, legal, and social implications of population-based genotyping are still unresolved and much debated. It is important that distinctions are made between disease susceptibility gene polymorphisms, which provide information about risks of diseases, and pharmacogenetic profiles (120), even though it is not always possible to make this distinction. An example is the ApoE polymorphism. This polymorphism might predict a patient's response to statins (76) or the risk for discontinuation of statins (86), but it also predicts a patient's risk on developing Alzheimer's disease (121). For such polymorphisms, it might lead to difficult decisions for health care professionals. Is it the task of health care professionals to tell the patient about this risk? The patient has of course the right (not) to know. This information might not only influence the patient, but also members of his family who might carry the same polymorphism. Furthermore, it might not only influence the patient's perception of his health and life, but also his eligibility for healthcare and life insurance. The debate is ongoing in various countries with, so far, an uncertain outcome.

If polymorphisms exist that influence the response to statins, it is also important to evaluate the cost-effectiveness of screening for such genotypes. In a recent study, the cost-effectiveness of screening for the ACE genotype before starting statin therapy was evaluated (122). If the interaction between the ACE genotype and the effectiveness of statin therapy is confirmed in more studies, then screening could save money. The model used in this study can also be used to determine the cost-effectiveness for screening for other polymorphisms.

At present, no single polymorphism has been identified that renders statin treatment ineffective based on clinical outcomes. Therefore, results from large-scale population studies are needed, to complement results from clinical trials and small-scale studies in selected populations.

Possibly, one day a person's genotype might determine if he or she should get a statin or not. Then, determining the genotype will not deny therapy to a subject, but will help in the decision as to which therapy suits the patient best, and potentially increase cost-effectiveness of the treatment

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## Monogenic Causes of Heart Failure

### *Familial Dilated Cardiomyopathy*

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#### SUMMARY

Dilated cardiomyopathy (DCM) without an established causative factor for the damage to the myocardium is termed “idiopathic DCM,” and it is the cause of approximately one-fourth of the cases of congestive heart failure in the United States. Familial occurrence accounts for 20% or more of idiopathic DCM, and recently a considerable number of genes associated with DCM have been identified. This review will focus on 18 genes currently known as *DCM response genes* because of their pathological involvement in this disease. The incidence of DCM caused by each gene is relatively small; however, the study of these genes has led us to understand the specific disease pathways of DCM, which may ultimately lead to understanding the more common condition of heart failure.

**Key Words:** Dilated cardiomyopathy; (DCM); gene; cardiomyocytes; familial; sarcomere; Z-disc; desmosome; costamere; adherens junction.

#### INTRODUCTION

Heart failure defined by Braunwald is the pathophysiological state in which the heart is unable to pump blood at a rate commensurate with the requirement of the metabolizing tissues (1). It is usually, but not always, caused by a defect in myocardial contraction. A number of adaptive mechanisms for maintenance of pumping function occur both rapidly and slowly: the Frank–Starling mechanism, in which an increased preload helps to sustain cardiac performance; activation of neurohumoral systems, especially the release of

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the norepinephrine, which augments myocardial contractility; and the activation of the renin–angiotensin–aldosterone system, which maintains arterial pressure and perfusion of vital organs. Myocardial remodeling develops over weeks to months, and plays an important role in long-term adaptation to hemodynamic overload. If compensatory responses are adequate to match the work demands, a period of relative stability ensues. However, if the extent or form of myocardial remodeling is insufficient, or if the magnitude of the overload increases, there is further deterioration in myocardial function leading to advanced heart failure (1).

Approximately 400,000 new cases of heart failure occur annually, and its prevalence demonstrates the enormous burden exacted on the public: 4.7 million patients and their direct health care cost \$17.8 billion in 1993 (2). Primary cardiovascular diseases including coronary atherosclerosis, hypertension, myocarditis, diabetes, valvular and congenital heart malformations, and cardiomyopathy lead to the common final pathway of heart failure. The cardiomyopathies constitute a group of diseases in which the dominant feature is direct involvement of the heart muscle, not the result of hypertensive, valvular and congenital heart malformations, or ischemic diseases. Dilated cardiomyopathy (DCM) is characterized by dilation and impaired contraction of the left or both ventricles, and is caused by a variety of cytotoxic, metabolic, immunological, infectious, and familial mechanisms. DCM without an established causative factor that damages the myocardium is termed “idiopathic DCM.” About one-fourth of the cases of congestive heart failure in the United States are the result of idiopathic DCM. Although the causes remain unclear, interest has been centered on three possible basic mechanisms: (1) familial and genetic factors, (2) viral myocarditis and other cytotoxic insults, and (3) immunological abnormalities (1).

Familial occurrence accounts for 20% or more of idiopathic DCM (1), and recently a considerable number of genes have been identified as causative factors for DCM. Although the detailed disease mechanisms caused by each mutation have not been elucidated, current general agreement is that familial DCM is likely a disease of the cytoskeleton leading to inadequate force transmission (3–6). In this review, 18 genes, currently known as DCM responsive genes, are focused on for their pathological involvement in DCM. The incidence of DCM caused by each gene is relatively small; however, these studies have led to a great understanding of the specific disease pathways of DCM that may ultimately lead to understanding the more common condition of heart failure.

## STRUCTURES OF THE CARDIAC CYTOSKELETON, CELL ADHESION, AND THE SARCOMERE

The major function of cardiomyocytes is in the cardiac contraction–relaxation cycle. The contractile proteins in cardiomyocytes constitute about 75% of the total volume of the myocardium, although only about one-third of the number of all the cells in the myocardium are cardiomyocytes. About half of each ventricular cell is occupied by myofibers, and about one-fourth to one-third by mitochondria. The major proteins involved in contraction and relaxation are the thin filament (composed of actin, tropomyosin, and C-, I-, and T-troponin) and the thick myosin filament. During contraction, the filaments slide over each other (A band) without the individual molecules of actin or myosin actually shortening, leading to a pulling together of the two ends of the fundamental contractile unit called the sarcomere (Fig. 1A,B). The sarcomere is limited on either side by the Z-disc (“Z” is an abbreviation for the German “Zückung”, meaning “contrac-

tion”) to which the actin filaments are attached. Conversely, the myosin filaments extend from the center of the sarcomere (M-line) in either direction toward, but not actually reaching the Z-discs.

The third component, titin (or connectin), is anchored in the Z-disc and extends to the M-line region of the sarcomere (Fig. 1A,B). Titin is an extraordinarily long, flexible, and slender myofibrillar protein that tethers myosin to the Z-disc to maintain the precise structural arrangement of thick and thin filaments (7,8). Titin’s I-band region appears to function as a molecular spring. When the sarcomere is stretched, this region is also extended and held away from the Z-disc, giving rise to the passive force that pulls Z-discs toward each other. When the sarcomere shortens, the thick filament moves into titin’s near the Z-disc region, leading to titin’s spring region being stretched in an opposite direction and generating the force that pushes the Z-disc away (Fig. 1B).

Contractile force generated within cardiac sarcomeres transmits to adjacent sarcomeres via the Z-disc (Fig. 1A,B), to neighboring myocytes via intercalated discs (desmosome, adherens junction) (Fig. 2A), and to the extracellular matrix via costameres (Fig. 2B). In DCM, it is likely that the structural integrity of force-transmitting proteins is compromised, leading to nonuniform myocyte contraction and increased vulnerability to myocellular injury and death under physiological mechanical stress.

## GENES IDENTIFIED AS RESPONSIBLE FOR IDIOPATHIC DCM

The genes that cause familial DCM are summarized in Table 1 according to their intracellular localization: Z-disc localizing proteins ( $\alpha$ -actinin-2, muscle LIM protein [MLP], Z-disc alternatively spliced PDZ motif [ZASP]/cypher, titin-cap [T-cap]/telethonin); costameres, adherens junctions, desmosomes, and intermediate filaments (vinculin/metavinculin, desmoplakin, plakoglobin, desmin, lamin A/C,  $\alpha$ -actinin-2, MLP, dystrophin,  $\delta$ -sarcoglycan); sarcomere proteins (cardiac  $\alpha$ -actin,  $\beta$ -myosin heavy chain, cardiac troponin T,  $\alpha$ -tropomyosin, titin); sarcoplasmic reticulum proteins (phospho-lamban); and ion channel (SUR2A).

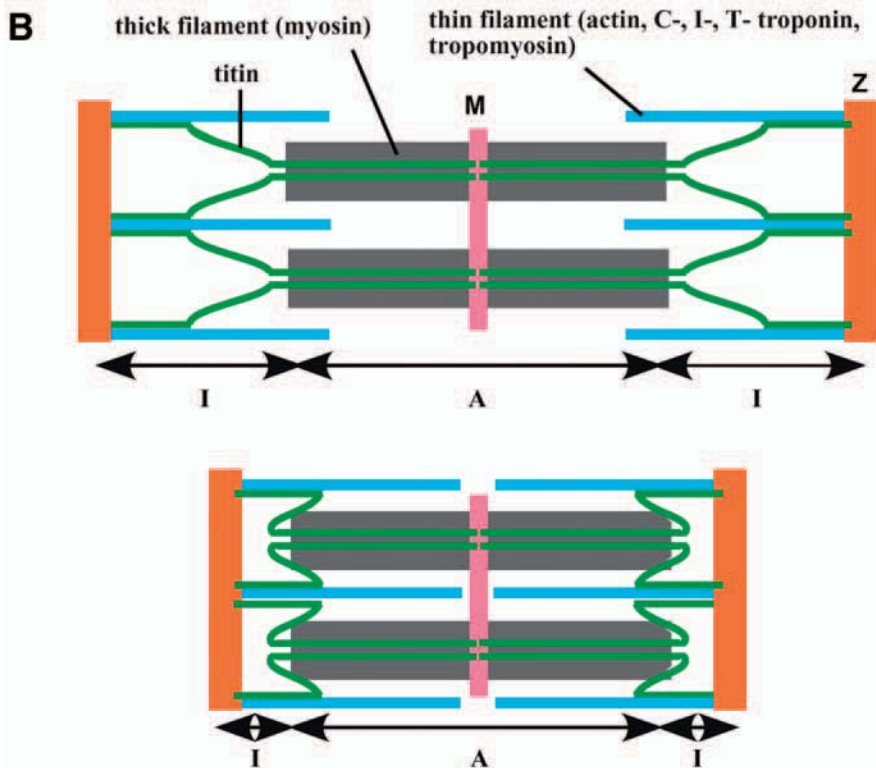
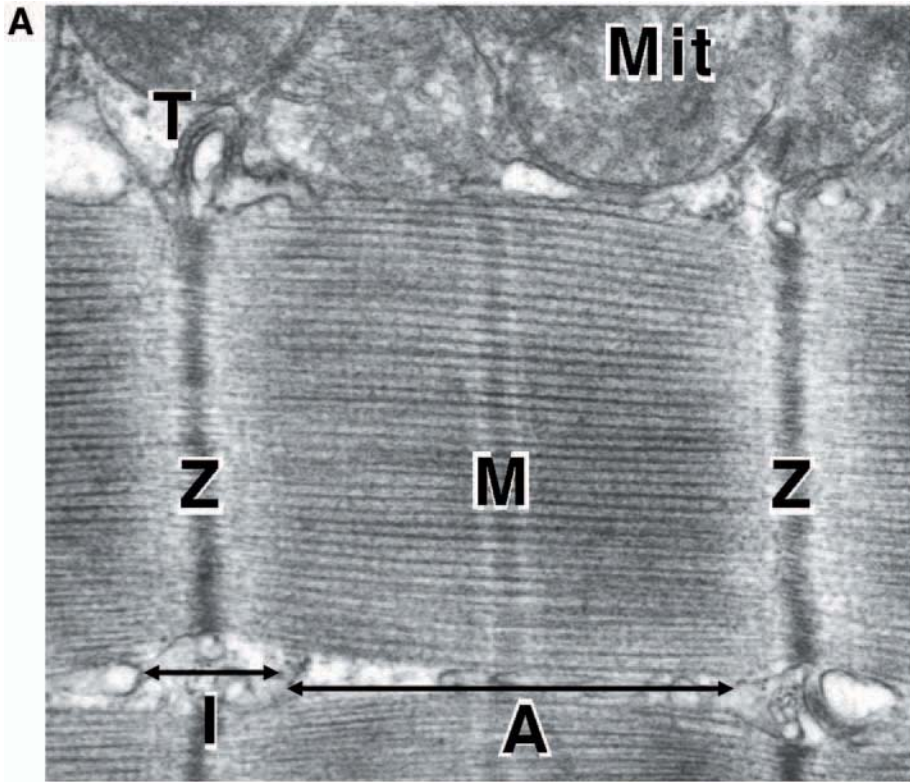
### *Titin and Z-Disc Proteins ( $\alpha$ -actinin-2, MLP, ZASP/Cypher, T-Cap/Telethonin)*

Both thin filaments (actin) and thick filaments (myosin through a titin) anchor at Z-discs. Therefore, the Z-disc is considered a sensor of contraction with other Z-disc localizing proteins to keep the normal integrity of the cardiac muscle (9).

#### **TITIN**

A giant protein, titin (or connectin) is the third most abundant protein of vertebrate striated muscle after myosin and actin (7,8). The titin molecule is a flexible filament that is more than 1  $\mu$ m long, spanning half the sarcomere from the Z-disc (amino terminus of titin) to the M-line (carboxyl terminus of titin) (Fig. 1B). The molecule is formed as a single polypeptide with a molecular weight of up to 3 MDa, and is the largest polypeptide found so far in nature (10). The titin molecule is predominantly composed of nearly 300 immunoglobulin-like domains and fibronectin type III repeats. An additional kinase domain near the carboxyl terminus and several potential phosphorylation sites have also been identified. Within the Z-disc, titin interacts with other Z-disc proteins: T-cap/telethonin at two amino terminal immunoglobulin-like domains and  $\alpha$ -actinin at the repeated unique 45 amino acid residues (Z-repeats), which crosslinks antiparallel thin





filaments from adjacent sarcomeres. The two immunoglobulin-like domains located carboxyl terminus to the Z-repeats associate with obscurin, a giant multimodular protein that probably has a signaling function. The relationship between titin and actin within the Z-disc region is not yet clear, but it is known that near the edge of the Z-disc, titin attaches to the thin filaments (Fig. 2C). The Z-disc part of titin is also likely to be physically linked to intracellular membrane systems, T-tubules, and the sarcoplasmic reticulum (SR). Association with the T-tubule is likely through T-cap/telethonin, which binds to T-tubule potassium channels (MinK). Links with the SR could involve either direct interactions between titin and ankyrin, or indirect interactions through obscurin, which could link titin with ankyrin.

The carboxyl end of titin binds to M protein and myomesin at the M-line. The I-band region of titin contains only immunoglobulin domains and has a unique sequence, whereas the A band contains immunoglobulin-like domains and fibronectin type III repeats in long-range patterns.

Two mutations of titin proteins have been identified in autosomal dominant DCM without cardiac conduction defects or clinically detectable skeletal muscle disease (11). One 2-basepair (bp) insertion mutation in exon 326 causes a frameshift, generating a premature stop codon. However, tissue lysates prepared from skeletal muscle of an affected individual demonstrated that the mutant protein is smaller than the predicted molecular weight and only proteolytic digested mutant proteins near the PEVK segment (enriched with proline [P], glutamate [E], valine [V], and lysine [K] residues), which is important for titin's spring in the I-band region of titin, are stably expressed. The other mutation, a single nucleotide substitution in exon 18, alters tryptophane to arginine within an immunoglobulin domain located near the Z-disc to I-band transition zone (11). Clear explanations for the functions of mutant proteins that dominantly affect only cardiac myocytes remain to be found, but these mutant proteins may disrupt the assembly of contractile filaments or decrease the elasticity of titin molecules.

### T-CAP/TELETHONIN

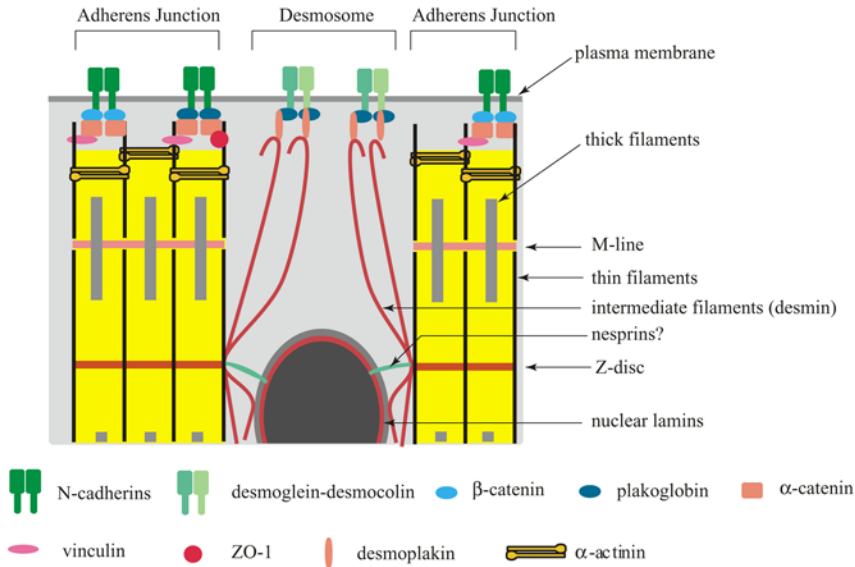
Nineteen-kDa of T-cap, or telethonin (12,13), interacts with the amino terminus of immunoglobulin-like domain of titin (Fig. 2C). T-cap/telethonin function has been suggested as important for organizing myofibrils because overexpression of T-cap/telethonin protein results in myofibril disruption, likely through inhibiting endogenous titin-T-cap/telethonin interaction at Z-discs.

T-cap/telethonin mutation was identified in a single DCM patient (Arg87Gln mutation). This mutation disrupts a stable complex formation with MLP (14). T-cap/telethonin

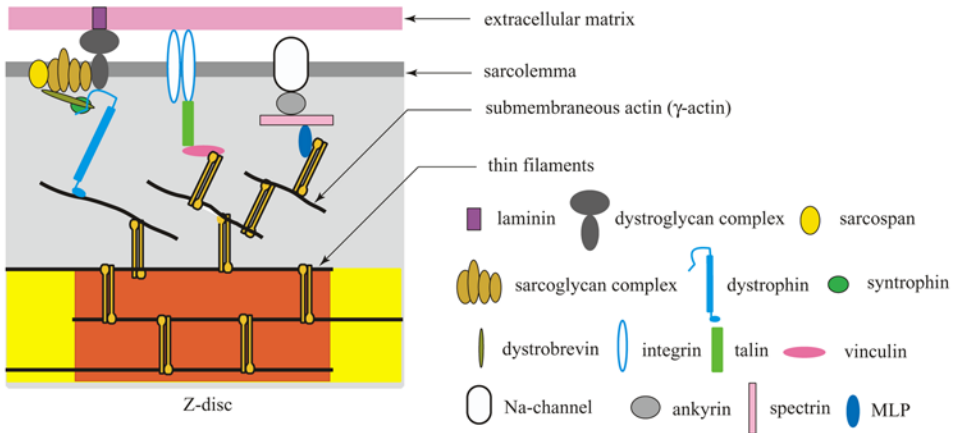
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**Fig. 1.** Structure of the sarcomere. (A) Thick filament myosin is attached to M line (M), and thin filament (composed with actin, tropomyosin, and C-, I-, and T-troponin) is attached to Z-disc (Z). Thin filaments and thick filaments overlap at A band (A) but no myosin at I band (I). T tubules (T) penetrate into the muscle at the level of the Z-line. Mit, mitochondria. EM from adult mouse heart. (B) The third component, titin (or connectin) anchors in the Z-disc and extends to the M-line region of the sarcomere. The titin's I-band region appears to function as a molecular spring. When the sarcomere is stretched (top panel), this region is also extended and held away from the Z-disc, giving rise to the passive force that pulls Z-discs toward each other. When the sarcomere shortens (bottom panel), the thick filament moves into titin's near Z-disc region leading to the titin's spring region being stretched in an opposite direction and generating the force that pushes the Z-disc away.

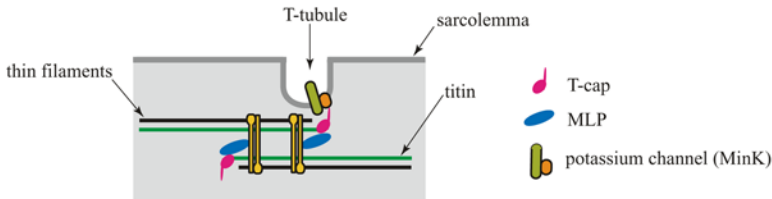
**A** Intercalated Disc: Desmosome and Adherens Junction



**B** Costamere: Dystrophin-glycoprotein complex, Integrin-based focal adhesion, Spectrin-based membrane cytoskeleton



**C** Z-disc and T-tubule



**Fig. 2.** Structure of desmosome and intermediate filaments, adherens junction, and costamere. (A) Two cells are attached at adherens junction and desmosome. Adherens junction is composed of transmembrane protein. (N-cadherin) and intracellular attachment proteins (including  $\beta$ -catenin,  $\gamma$ -catenin, vinculin, ZO-1 and  $\alpha$ -actinin). Actin filaments are attached to the adherens junction. Desmosome is composed of transmembrane protein, desmosomal cadherin, desmoglein, and desmocolin, and intracellular attachment proteins (including desmoplakin and plakoglobin). Thick intermediate

mutations were also identified in autosomal recessive limb-girdle muscular dystrophies type 2G in which some patients show a cardiac phenotype (15).

In addition, T-cap/telethonin associates with MinK,  $\beta$ -subunit of slow activating component of the delayed rectifier potassium current channel ( $I_{Ks}$ ) (16). This interaction may contribute to a stretch-dependent regulation of potassium flux in cardiac muscle, providing a mechano-electrical feedback system. The T-tubule is located at the Z-disc in cardiomyocytes, whereas it is located at the A-I junction in mammalian skeletal muscle. The different T-tubule localization between cardiac and skeletal muscle may indicate the importance of physical interactions between ion channels on the T-tubule and Z-disc proteins in cardiomyocytes.

### MUSCLE LIM PROTEIN

MLP is a striated muscle-specific factor of the cysteine-rich protein (CRP) family that enhances myogenic differentiation and is critical for maintaining structural integrity. MLP protein is composed of two tandem LIM domains followed by a glycine-rich region. Each LIM domain contains double zinc finger-like structures that serve as a protein-binding interface. The first LIM domain seems to be important for binding to  $\alpha$ -actinin (14), the second LIM motif of MLP can associate with  $\beta$ -spectrin (Fig. 2B) (17), and the short fragment (5 amino acids) at the amino terminus of MLP is critical for interactions with T-cap/telethonin (14). In addition to Z-disc accumulation, MLPs are localized in the nucleus and regulate transcription as a cofactor of transcription factors, including myoD in the skeletal myocytes (18,19).

Among 536 DCM patients, 10 patients show an autosomal dominant mutation at the amino terminus of MLP (Trp4Arg) that abolish the interaction with T-cap/telethonin (Fig. 2C) (14). In normal neonatal cardiomyocytes, both mechanical stretch as well as the  $\alpha$ -adrenergic agonist phenylephrine induce expression of the hypertrophic gene marker, brain natriuretic peptide (BNP). In MLP-null cardiomyocytes, phenylephrine induced BNP expression, whereas mechanical stretch failed to induce BNP expression, suggesting specific defects in mechanosensing pathways for BNP induction in MLP-null cardiomyocytes. DCM mutations in T-cap/telethonin and in MLP are found in the region critical for interaction of the two proteins, indicating that the T-cap/telethonin-MLP interaction is important for a mechanical stress sensor pathway (14). Another mutation in MLP (Lys69Arg) adjacent to the first LIM domain was found in DCM patients (20). This mutation abolished the interaction with  $\alpha$ -actinin; however, three other MLP mutations identified in hypertrophic cardiomyopathy (HCM) patients were within the same LIM domain that is critical for binding to  $\alpha$ -actinin (21). Because MLP-knockout mice

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**Fig. 2.** (continued from opposite page) filaments (desmin) are attached to the desmosomes, which will connect to sarcomere at the Z-disc. Another intermediate filament comprising inner nuclear membrane, nuclear lamins are also connecting to Z-disc through nesprin proteins. Nuclear membrane structure is composed of nuclear pore complex, outer nuclear membrane, and inner nuclear membrane, to which heterochromatin is attached. **(B)** Cell-substrate interactions are mediated by costameres at the sarcolemma overlying the Z-discs. At costameres, multiple unique protein complexes link among the extracellular matrix, sarcolemma, and intracellular cytoskeleton, including actin and intermediate filaments. Three major protein complexes at the costameres are known: integrin-talin-vinculin- $\alpha$ -actinin complex (1-4), Na-channel-ankyrin-spectrin- $\alpha$ -MLP-actinin complex, and dystrophin-glycoproteins complexes. **(C)** Protein interactions among titin, T-cap/telethonin, MLP,  $\alpha$ -actinin, and potassium channel (MinK) at Z-disc and T-tubule.

Table 1  
Genes Causing Familial DCM

<i>Group</i>	<i>Gene/protein</i>	<i>Chromosomal locus</i>	<i>OMIM no.</i>	<i>Inheritance pattern</i>	<i>Additional phenotype</i>
Z-disc proteins	ZASP/cypher	10q22.3-q23.2	605906	AD	Isolated noncompaction of the left-ventricular myocardium
	$\alpha$ -actinin-2 <i>MLP</i>	1q42-q43 11p15.1	102573 607482	AD AD	
Costameres and adherens junction Desmosome and intermediate filaments Dystrophin-sarcoglycan	<i>T-cap/telethonin</i>	17q12	604488	AR	LGMD 2G
	Metavinculin/vinculin $\alpha$ -actinin-2	10q22.1-q23 1q42-q43	193065 102573	AD AD	
	<i>MLP</i>	11p15.1	607482	AD	
	Desmoplakin Plakoglobin	6p24 17q21	125647 601214	AR AR	Woolly hair, keratoderma, ARVD/C Naxos disease, ARVD/C, woolly hair, keratoderma
Sarcomeric proteins	Desmin	2q35	125660	AD, AR, <i>de novo</i>	Desmin myopathy
	Lamin A/C	1q21.2	607920, 150330	AD	Conduction disease, skeletal myopathy
	Dystrophin	Xp21	300377, 302045	X	Skeletal myopathy
	$\delta$ -sarcoglycan	5q33	601411, 606685	AD	LGMD 2F
	Cardiac $\alpha$ -actin	15q14	102540	AD	
	Cardiac troponin T $\beta$ -myosin heavy chain $\alpha$ -tropomyosin	1q32 14q12 15q2	601494, 191045 160760 191010	AD AD AD	
Sarcoplasmic reticulum protein Ion channel	Titin	2q31	18840	AD	Familial HCM; tibial muscular dystrophy
	Phospholamban	6q22.1	172405	AD	Ventricular tachycardia
	<i>SUR2A</i>	12p12.1	601439	AD	

DCM, dilated cardiomyopathy; OMIM, Online Mendelian Inheritance in Man; LGMD, limb-girdle muscular dystrophy; HCM, hypertrophic cardiomyopathy; MLP, major latex protein; ZASP, Z-band alternatively spliced PD2 motif.

show both hypertrophic and dilated cardiomyopathic features (22), MLP mutations may cause both DCM and HCM at different stages.

Additional potential MLP functions can be observed by interacting with  $\beta$ -spectrin through the second LIM domain of MLP.  $\beta$ -spectrin is localized both at the sarcomere and costamere (17). Costameres, submembranous transverse ribs found in many cardiac muscle cells, are thought to anchor the Z-discs of the peripheral myofibrils to the sarcolemma (Fig. 2B). It has been postulated that costameres provide mechanical linkage between the cells' internal contractile machinery and the extracellular matrix (23).

#### **$\alpha$ -ACTININ-2**

$\alpha$ -actinin is an actin-binding protein with multiple roles in different cell types. In nonmuscle cells, the cytoskeletal isoforms ( $\alpha$ -actinin-1 and -4) are found along microfilament bundles and adherens junctions, where actinin is involved in actin and membrane interactions. In contrast, skeletal, cardiac, and smooth muscle isoforms ( $\alpha$ -actinin-2 and -3) are localized to the Z-disc, where they help anchor the myofibrillar actin filaments (Fig. 2A). In humans,  $\alpha$ -actinin-2 is widely expressed in skeletal and cardiac myocytes, whereas  $\alpha$ -actinin-3 is expressed only in skeletal muscle and is absent in 18% of normal human populations (24).

One mutation identified in DCM patient substitutes glutamine 9 to arginine. Wild-type  $\alpha$ -actinin-2 binds to MLP, whereas it was abolished in Gln9Arg mutants. This result suggests the importance of  $\alpha$ -actinin-2 and MLP interaction in forming mechano-sensing complexes (20).

#### **ZASP/CYPHER**

ZASP, or cypher, is a member of ALP-enigma family containing one amino terminal PDZ domain, and one or three carboxyl-terminal LIM domains. Most of the members including enigma, ENH, ALP, and Ril associate with the cytoskeleton, such as  $\alpha$ -actinin and/or  $\beta$ -tropomyosin with their PDZ domain, and are localized at the Z-disc (25,26). ZASP/cypher-null mice showed congenital myopathy resulting in a gasping respiratory pattern, limb weakness, and a limited range of motion, and the majority of mice die within 24 h (27). Cardiac muscle ZASP/cypher-null mice display fragmented and disorganized Z-discs, suggesting that ZASP/cypher is essential to support Z-disc structure by linking with  $\alpha$ -actinin.

ZASP/cypher mutations are found in human patients with DCM with or without isolated noncompaction of the left ventricular myocardium. Vatta et al. reported 6 ZASP/cypher mutations among 100 patients (28), in which five are missense mutations within the linker between PDZ and LIM domain, likely to form different secondary structures. Indeed, C2C12 cells transfected with ZASP/cypher with one mutation, Asp117Asn, showed abnormal ZASP/cypher and actin staining. Arimura et al. reported 1 among 96 patients (29). The mutation was in the third LIM domain, which increases the interaction with protein kinase C, suggesting the involvement of intracellular signaling in disease mechanisms.

### ***Desmosome, Adherens Junction, Costamere, and Intermediate Filaments***

In the heart, cell–cell and cell–substrate interactions are mediated by specific structures: desmosome, adherens junction, and costamere (Fig. 2A,B). These structures are important for holding cells together under the influences of the extracellular matrix and

for their development, migration, proliferation, shape, and function. Desmosomal cell–cell junctions are found predominantly in the heart and skin. In the heart, desmosomes are localized at the intercalated disc. They link the intermediate-size filament networks (such as desmin in the heart) to the cell membrane to maintain tissue architecture and integrity. The desmosome consists of several proteins, which can be divided into two groups: the urea-insoluble “core” and the urea-soluble “plaque” components. The main proteins of the plaque comprise the desmoplakins and plakoglobins ( $\gamma$ -catenin) that are responsible for connecting the cytoskeleton to the transmembrane adhesion proteins, desmoglein and desmocollin which belong to the cadherin family and fractionate into the urea-insoluble “core” (30).

Adherens junctions are localized at the intercalated disc and contain the “classical” cadherin cell adhesion molecules, N-cadherin, of which the extracellular domains mediate  $\text{Ca}^{2+}$ -dependent homophilic adhesion (Fig. 2A). Cytoplasmic domains of N-cadherin binds to  $\beta$ -catenin or plakoglobin ( $\gamma$ -catenin) and mediate the anchorage of the cadherin– $\beta$ -catenin/plakoglobin complex to the actin cytoskeleton through  $\alpha$ -actinin, either directly interacting with F-actin or indirectly through the actin-binding proteins vinculin,  $\alpha$ -actinin, and ZO1 (30).

Costameres are rib-like bands at the sarcolemma overlying the Z-discs (Fig. 2B). At costameres, multiple unique protein complexes link among the extracellular matrix, sarcolemma, and intracellular cytoskeleton, including actin and intermediate filaments. Three major protein complexes at the costameres are known: integrin–talin–vinculin– $\alpha$ -actinin complex, Na-channel–ankyrin–spectrin–MLP– $\alpha$ -actinin complex, and dystrophin–glycoproteins complexes (31).

Intermediate filaments are tough and durable protein fibers found in the cytoplasm of most cells. They are called “intermediate” because in electron micrographs their apparent diameter (8–10 nm) is between that of the thin actin filaments and the thick myosin filaments of muscle cells, where they were first described (they are also intermediate in diameter between actin filaments and microtubules). In most animal cells, an extensive network of intermediate filaments connects among the nucleus, contractile proteins (by surrounding the Z-discs), sarcolemma (Fig. 2A), and extracellular matrix via costameres (Fig. 2B). They also have the potential to associate with other organelles including mitochondria, and the SR.

#### VINCULIN/METAVINCULIN

Vinculin and metavinculin are components of adherens junctions as well as costameres (Fig. 2A,B). Vinculin and metavinculin contain binding domains for multiple cytoskeletal proteins, including actin,  $\alpha$ -actinin, talin, paxillin, vasodilator-stimulated phosphoprotein, ponsin, vinexin, and protein kinase C. Their head and tail regions physically interact in a resting state to mask most binding sites. By exposure to phosphatidylinositol (4,5)-bis-phosphate (PIP<sub>2</sub>), the open “activated” conformation of vinculin exposes all binding sites (32). The vinculin gene is located on chromosome 10q22.1-q23 and comprises 22 exons. Exon 19 is alternatively spliced in a tissue-specific manner, and the smaller isoform with a shorter tail, vinculin (116 kDa), is ubiquitously expressed, whereas metavinculin, the larger isoform with a longer tail containing an additional 68 amino acids, is expressed exclusively in cardiac and smooth muscle. In cardiac myocytes, vinculin and metavinculin are coexpressed at intercalated discs and costameres.

An absence of metavinculin transcripts and protein combined with normal vinculin expression was detected in one DCM patient (33). Another study showed two mutations,

a single missense mutation (Arg975Trp), and a 3-bp deletion (Leu954del), in the metavinculin-specific exon 19 among 350 DCM patients (34). A Z-disc abnormality was detected in both cases, suggesting a metavinculin-specific role in maintaining Z-disc structure uncompensated by vinculin. The tail domains of vinculin and metavinculin both bind to F-actin. Vinculin forms large needle-like structures with actin, whereas metavinculin form viscous webs with actin. Different actin organization may arise as a result of a longer tail domain of metavinculin, making it more flexible to bind at crossover points of actin filaments and preventing parallel arrangements seen in vinculin (35).

#### **DESMOPLAKIN, PLAKOGLOBIN ( $\gamma$ -CATENIN)**

Desmoplakin and plakoglobin are components of desmosome (Fig. 2A). Recessive mutations in the desmoplakin gene cause a generalized striated keratoderma, particularly affecting the palmoplantar epidermis, resulting in woolly hair and leading to a dilated left ventricular cardiomyopathy that progresses to heart failure in the teenage years (36). The mutation identified in three families produces a premature stop codon, leading to a truncated desmoplakin protein missing the carboxyl-terminal domain which is important for interacting with the intermediate filaments network. Homozygous desmoplakin-null mice die in early embryogenesis (E 6.5) (37), whereas human recessive mutations (homozygous mutations) in the desmoplakin gene are not embryonically lethal, suggesting that the truncated desmoplakin molecule has some residual desmosomal function that is able to maintain the intermediate filament network without the desmoplakin tail domain unless it is stressed. However, in the restricted areas, such as the epidermal sites prone to pressure or in the continuously beating heart, the mutant desmoplakin may not be able to maintain tissue architecture.

In contrast, an autosomal dominant mutation at the aminoterminal domain of desmoplakin was found in arrhythmogenic right ventricular cardiomyopathy (ARVD/C), a progressive disease characterized by degeneration of the right ventricular myocardium, followed by fibrous-fatty replacement (38). ARVD/C usually clinically presents with arrhythmias of right ventricular origin, ranging from isolated premature ventricular beats to sustained ventricular tachycardia or to ventricular fibrillation leading to sudden death. The aminoterminal region of desmoplakin is required for localization of desmoplakin to the desmosomal plaque by binding to desmoplakin itself, desmocollin, and plakoglobin. Plakoglobin gene is also mapped to the ARVD/C disease locus (Naxos disease). The involvement of two desmosomal proteins in ARVD/C suggests that some ARVD/C might result from defects in intercellular connections.

Plakoglobin ( $\gamma$ -catenin) is a component of desmosome, as well as the adherens junction (Fig. 2A) at which the cadherin–catenin complex anchors to actin filaments. Plakoglobin-null mice die around E12–16 due to defects in heart function often associated with ventricular rupture and blood floods in the pericardium (39). Hearts from plakoglobin-null mice demonstrate no desmosome formation, instead extended adherens junctions develop, which contain desmosomal proteins such as desmoplakin. These results suggest that plakoglobin is not only essential for the formation of stable cardiac desmosomes, but is also critically involved in the segregation and/or sorting of the molecules into desmosome and adherens junction.

#### **INTERMEDIATE FILAMENT**

Intermediate filament, desmin (40,41), and nuclear lamin A/C mutations (Fig. 2A) are also identified in DCM patients (40,41).



## DESMIN

Desmin is encoded by a single copy gene located in chromosome 2q35. A 53-kDa protein interacts with other intermediate filament proteins to form an intracytoplasmic network that maintains spatial relationships between the contractile apparatus and other structural elements of the cell. Twenty-one pathogenic mutations have been identified in desmin, and the current understanding of disease mechanisms is that mutant desmin proteins are unable to properly assemble into normal filaments. The desmin-null mice are viable and fertile. However, these mice demonstrated a multisystem disorder involving cardiac, skeletal, and smooth muscle. Histological and electron microscopic analyses in both heart and skeletal muscle revealed severe disruptions of muscle architecture and degeneration. Structural abnormalities included loss of lateral alignment of myofibrils and abnormal mitochondrial organization (42).

## LAMIN A/C

The nuclear envelope consisting of the inner membrane, outer membranes, and nuclear pore complexes organizes nuclear architecture and separates the chromosomes from the cytoplasm. The inner membrane is a network of an intermediate filament protein, lamins, that faces the chromosomes and anchors several membrane proteins such as the lamin B receptor, lamin-associated protein-1 (LAP-1), and emerin (Fig. 2A). At least three lamin genes—*A*, *B1*, and *B2*—identified in humans are differentially expressed during development. B-type lamins are expressed widely, whereas lamin A protein is expressed primarily, but not exclusively, in differentiated nonproliferating cells. Lamins *A* and *C* are the alternatively spliced forms encoded by a single gene, and both contain the identical first 566 amino acids with 98 unique carboxyl-terminal amino acids for lamin A and 6 unique amino acids for lamin C (43).

Fatkin et al. first reported five missense mutations among 11 families with autosomal dominant DCM with conduction defects (44). Since then, up to 40 mutations have been reported (45). Lamin *A/C* mutations are identified in several different inherited diseases, including cardiomyopathy with variable skeletal muscle dystrophy (dilated cardiomyopathy with conduction defects, autosomal dominant and recessive Emery–Dreifuss muscular dystrophy, limb-girdle dystrophy type 1B), peripheral neuropathy (Charcot–Marie–Tooth disorder type 2B1), and partial lipodystrophy syndromes (46). Lamin A has two globular domains (N- and C-terminus) and a central  $\alpha$ -helical region. The positions of mutation-causing DCM are scattered in lamin A protein and a broad spectrum in the severity of phenotypes within family members carrying the same mutation are observed (47–49).

The disease mechanisms of lamin *A/C* mutations have been explained by two nonexclusive hypotheses: the “mechanical stress” hypothesis, and the “gene expression” hypothesis (46). The mechanical stress hypothesis states that abnormalities in nuclear structure resulting from lamin mutations lead to increased susceptibility to cellular damage by physical stress, based on the abnormalities in nuclear morphology observed in both human patients and animal models. The gene expression hypothesis states that the nuclear envelope plays a role in tissue-specific gene expression that can be altered by mutations in lamins, primarily based on known interactions between the nuclear envelope and chromatin components.

Animal models of lamin *A/C* mutations have been examined in both lamin *A/C*-null mice and Leu 530 Pro knock-in mice, a mutation identical to that which causes autosomal dominant Emery–Dreifuss muscular dystrophy (50,51). Heterozygous Leu 530 Pro

knock-in mice do not show signs of muscular dystrophy; however, homozygous Leu 530 Pro mice show growth retardation and death by 4 wk, with premature aging. Mutant hearts are smaller with decreased number of cardiomyocytes, suggesting either incomplete heart development or loss of muscle mass. Lamin A/C-null mice also show growth retardation and muscular dystrophy, and die by 8 wk.

Recently, a connection between lamin A and the cytoskeleton has been reported through a newly identified protein, nesprin-1 and -2 (Syne-1/myne-1, Syne-2), nesprin is a large protein containing actin-binding calponin homology at its amino-terminus and many spectrin repeats at the central region. Specific antibodies against the amino-terminus region (residue 5470–5485) revealed a predominantly cytoplasmic distribution with localization to the Z-discs, whereas the antibody against the carboxyl-terminus region (residue 8689–8704) revealed a strong localization at the nuclear envelope in association with lamin A and emerin (52). Mutations in the *Caenorhabditis elegans* homolog of nesprin, ANC-1 resulted in freely floating nuclei within the cytoplasm and nuclei formed clusters in the hypodermal cells that contain more than 100 nuclei, which are normally uniformly spaced. This study suggests an important role of nesprin in the process of nuclear positioning in the cytoskeleton (46,53–55).

#### **DYSTROPHIN–SARCOGLYCAN COMPLEX**

Dystrophin is a large, rod-shaped protein with a molecular weight of 427 kDa that comprises four domains. The amino-terminal domain has homology with  $\alpha$ -actinin, and contains between 232 and 240 amino acid residues depending on the isoform. The central rod domain is a succession of 25 triple-helical repeats similar to spectrin, and contains about 3000 residues and a cysteine-rich domain of 280 residues which bind to  $\beta$ -dystroglycan. The last carboxyl-terminal domain comprises 420 residues, which bind to syntrophin and  $\alpha$ -dystrobrevin. The protein is associated with the plasma membrane of cardiac and skeletal muscle (sarcolemma), and its main role at the sarcolemma is to interact with integral membrane proteins (sarcoglycans, dystroglycans, syntrophins, and dystrobrevin complexes) that are assembled in the dystrophin–glycoprotein complex (Fig. 2B) (56). This complex forms a bridge across the sarcolemma and flexibly connects the basal lamina to the extracellular matrix to the inner cytoskeleton, F-actin. The main roles of the dystrophin–glycoprotein complex are to stabilize the sarcolemma and to protect muscle fibers from long-term, contraction-induced damage and necrosis, and to have a role in cellular communication by acting as a transmembrane signaling complex (57).

Mutations in the dystrophin gene cause Duchenne and Becker muscular dystrophies. Although cardiac involvement is invariably associated with Duchenne and Becker muscular dystrophies, rare mutations cause an almost exclusive cardiac involvement. According to Ferlini et al., the pathogenesis underlying the selective cardiac muscle impairment in the X-linked dilated cardiomyopathy families is not clear, although two main regions of the dystrophin gene appeared to be most commonly involved in X-linked dilated cardiomyopathy: the 5' end of the gene and the central hot-spot region, centered around exon 48–49 among a total of 79 exons (58). Mutations in the 5' end of the dystrophin gene likely affect splicing, leading to cardiac-specific transcriptional regulation of the dystrophin gene. On the other hand, mutations surrounding exons 48–49, including intron 48, may contain sequences very relevant for dystrophin function or expression mainly in the heart.

The sarcoglycan complex is a group of single-pass transmembrane proteins ( $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan) expressed in skeletal and cardiac muscle. The sarcoglycans are typically found in a heterodimeric complex including at least four different sarcoglycans. Although

the exact function of the sarcoglycan complex is not known, it is well established that mutations in any of  $\alpha$ -,  $\beta$ -,  $\gamma$ -, and  $\delta$ -sarcoglycan genes result in distinct forms of muscular dystrophy (59).

$\delta$ -sarcoglycan mutations cause autosomal recessive limb-girdle type muscular dystrophy (60). In contrast, autosomal dominant single missense mutations in  $\delta$ -sarcoglycan were reported in familial DCM without skeletal muscle abnormalities (61,62). Involvement of  $\delta$ -sarcoglycan in cardiomyopathy has been studied for several decades in the BIO14.6 Syrian cardiomyopathic hamsters, which have an approx 30-kb deletion in the regulatory region of the  $\delta$ -sarcoglycan gene leading to loss of  $\delta$ -sarcoglycan expression in cardiac and skeletal muscle (63,64). The BIO14.6 hamster carries a recessive mutation that leads to a degenerative disease of both heart and skeletal muscle. Although there are several sublineages of cardiomyopathic hamsters bred to have either dilated or hypertrophic cardiomyopathy, the pathogenetic mutation in  $\delta$ -sarcoglycan gene is identical. Hypertrophy in HCM hamsters is considered a compensatory reaction, and DCM hamsters might have another generic defect in this hypertrophic compensatory mechanism (63).

Recently, vascular spasm, especially in the coronary artery, resulting in focal cardiac ischemia has been reported in both hamsters and  $\gamma$ - and  $\delta$ -sarcoglycan knock-out mice (65,66). Transgenic expression of  $\gamma$ - or  $\delta$ -sarcoglycan using the  $\alpha$ -myosin heavy chain promoter, restoring cardiomyocyte expression of these proteins, rescued vascular spasm in  $\gamma$ - or  $\delta$ -sarcoglycan knockout mice, whereas SM-22-driven transgenic protein in vascular smooth muscle cells did not rescue vascular spasm. These observations suggest that cytokine release (presumably from vascular smooth muscle cells) accompanied by cardiomyocyte degeneration from lack of  $\gamma$ - or  $\delta$ -sarcoglycan can produce vascular spasm, which can lead to further cardiomyocyte damage resulting from focal cardiac ischemia.

### ***Sarcomeric Proteins/ $\alpha$ -Tropomyosin, $\beta$ -Myosin Heavy Chain, Cardiac Troponin T, Cardiac $\alpha$ -Actin***

Another group of DCM-responsive genes are sarcomeric proteins, including  $\alpha$ -tropomyosin,  $\beta$ -myosin heavy chain, cardiac troponin T, and cardiac  $\alpha$ -actin, which are all well-established causes of HCM rather than DCM. Many detailed reviews are available in HCM (6,67–69), therefore only the underlying pathological differences between HCM and DCM will be discussed here.

HCM mutations in sarcomere proteins were identified as early as 1990 (70). In addition, approx 60% of HCM populations are associated with mutations in sarcomeric proteins. Therefore, genotype–phenotype relations, as well as pathophysiology, are better understood in HCM than DCM. The pathogenesis of HCM has been explained by incorporation of mutant sarcomeric proteins, resulting in contractile dysfunction and subsequent activation of neuroendocrine and mechanical responses leading to compensatory hypertrophy. However, recent studies demonstrate that mutant sarcomeric proteins have enhanced motor activity with inefficient sarcomeric adenosine triphosphate (ATP) utilization, leading to a new energy-depletion hypothesis (69,71). Several animal models of reduced myocardial energy production reveal HCM phenotypes, including mitochondrial tRNA mutations, fatty acid uptake defects in CD36 deficiency, mitochondrial fatty acid oxidation defects in acyl-CoA dehydrogenase deficiency, adenine nucleotide translocator-1 knockout, which disrupts ATP export from mitochondria, as well as knockout of AMP-activated protein kinase (AMPK), which is both a sensor and critical orchestrator of cellular energy homeostasis.

In contrast, mutations found in sarcomere proteins in DCM in many respects oppose the data presented for HCM. For example, Arg92Gln troponin T mutation found in HCM patients shows increased  $\text{Ca}^{2+}$  sensitivity, ATPase activity, and thin-filament sliding velocity. In contrast, 3 nucleotides-deletion leading to a single amino acid deletion in Lys210 ( $\Delta$ 210) of troponin T mutation found in DCM patients is in the region responsible for  $\text{Ca}^{2+}$ -sensitive troponin C binding (72). This mutation is expected to reduce  $\text{Ca}^{2+}$ -stimulated actomyosin ATPase activity leading to reduced contractility, which was proven at least in vitro (73).

Four  $\beta$ -myosin heavy chain gene mutations were identified in DCM patients—Ala223Thr, Ser532Pro, Ser642Leu, or Phe764Leu amino acid substitutions—and none of them were found in HCM patients (72,74). Myosin–actin interactions for sliding movement occur with the head portion of myosin protein. Ser532 and Ser642, located in the head portion of myosin protein, contribute to the tight binding of actin; therefore, Ser532Pro and Ser642Leu mutations are expected to disrupt interactions between myosin and actin that are critical for initiating the power stroke of contraction. Phe764 locates in the hinge between the myosin head and rod part, suggesting its role for determining the magnitude or polarity of the head movement, and Phe764Leu mutation is suspected to reduce the efficiency of contraction. Ala223Thr mutation, locating close to the ATP-binding site, may decrease thermostability or affect the protein folding to change protein motility (74).

HCM mutation in the actin gene is involved in the direct actin–myosin interface, whereas in DCM, a Glu361Gly mutation in the actin gene is located at the actinin-binding domain important for force transmission to adjacent sarcomeres and neighboring myocytes (75).

Tropomyosin is one of five proteins comprising thin filaments of sarcomeres. Thin filaments play a dual role in force dynamics by regulating contractile force generation through thick filament (myosin) interactions and transmitting force through filament ends anchored to Z-bands and intercalated discs. Two missense mutations in the  $\alpha$ -tropomyosin gene, *Glu40Lys* and *Glu54Lys*, were reported as DCM-causative mutations that are different from HCM tropomyosin mutations (Ala63Val, Lys70Thr, Asp175Asn, Glu180Gly) (76). Because the surface of tropomyosin is dominated by negative charge, altered electrical charge resulting from a glutamate-to-lysine mutation observed in DCM patients may change electrostatic interactions between actin and tropomyosin filaments (76).

### *Sarcoplasmic Reticulum Proteins*

#### **PHOSPHOLAMBAN**

Phospholamban, a small, 6-kDa pentameric protein, comprises polypeptide subunits 52 amino acids in length. The nonphosphorylated form of phospholamban is an inhibitor of sarcoendoplasmic reticulum calcium pump (SERCA) 2a, but inhibition is relieved upon phosphorylation of phospholamban by  $\beta$ -adrenergic stimuli through cAMP-dependent protein kinase and  $\text{Ca}^{2+}$ -dependent calmodulin kinase. The subsequent activation of SERCA leads to enhanced muscle relaxation rates and an increased cardiac contraction rate and force by releasing  $\text{Ca}^{2+}$  from SR through the ryanodine receptor.

Two different types of phospholamban mutations were identified in DCM patients. Schmitt et al. reported an autosomal dominant DCM with C25T point mutation (causing Arg9Cys substitution). Arg9Cys mutant phospholamban appears to sequester protein kinase A from wild-type phospholamban, leading to increased levels of unphosphorylated

forms of phospholamban (77). Haghghi et al. reported T116G point mutation (causing early termination Leu39-stop) in two independent families. Patients with heterozygous mutation of Leu39-stop exhibited hypertrophy without diminished contractile performance, whereas patients with homozygous mutations developed dilated cardiomyopathy and heart failure (78).

Accordingly, two mutations will cause opposite effects: an Arg9Cys mutation will increase the level of dephosphorylated phospholamban, therefore SERCA 2a function should be inhibited; whereas a Leu39-stop mutation will cause a loss of phospholamban, therefore SERCA 2a function should be disinhibited (that is, activated). The consequent phenotype was not explained clearly, but these studies suggest the importance of balanced SERCA 2a activity that is both inhibited as well as augmented by phospholamban activity for normal heart function. Of note, phospholamban-null mice with increased SERCA 2a activity display chronically increased basal cardiac contractile function without developing heart failure even with advanced age, which is radically different from the DCM phenotype in human patients (79).

### *Ion Channel*

A recent study demonstrated a new DCM-causing mutant protein of sulfonylurea receptor-2 ([SUR2A]; sulfonylureas are widely used as oral hypoglycemic reagents in the treatment of noninsulin-dependent diabetes mellitus), a catalytic subunit of  $K_{ATP}$  ion channel protein (80). Cardiac  $K_{ATP}$  channels are composed of Kir6.2 and a regulatory subunit, SUR2A. One frameshift and one missense mutation were identified at the carboxyl-terminus of SUR2A, and are predicted to distort ATP-dependent channel pore regulation. Kir6.2-null mice developed arrhythmia and sudden death following sympathetic stimulation, which was preventable with calcium-channel blockers. The adrenergic stimuli enhance cardiac performance by increased  $Ca^{2+}$  handling, both inward and outward flow, which appears defective in  $K_{ATP}$ -null hearts (81).

### *Frequency of the Genetic Mutations in Familial and Sporadic DCM*

Although the prevalence of mutations in DCM described previously will be influenced by the patient population, several studies show that the causes of DCM are heterogenetic. Defects in  $\delta$ -sarcoglycan were found in two among 50 young DCM patients (aged 3 d–18 yr) (61), and two among 52 DCM patients (mean aged 50, age 11–71 yr) in Finnish patients. In the later study, the desmin gene and the metavinculin-specific exon of the vinculin gene were also examined in the same group, but no mutations were identified in these two genes (62). Also, the same author reported that no actin gene mutations were detected in 32 DCM patients (82). Apparently, only some of the known genes were screened in these studies, making it difficult to estimate the contribution of the known gene in DCM patients without screening all of the genes.

A recent study by Mohapatra et al. examined a total of 291 individuals with DCM, including 110 probands for the mutations in a total of 9 genes including cardiac actin, desmin,  $\delta$ -sarcoglycan, lamin A/C, troponin T, metavinculin,  $\alpha$ -dystrobrevin (83), MLP, and  $\alpha$ -actinin-2 (20). The contribution of these nine known genes in DCM patients seems relatively small; MLP mutations were found in two families, and  $\alpha$ -actinin mutations were found in one family.

## OVERVIEW

According to the list of DCM-related genes, three major categories of genes are dominant in the disease mechanism: Z-disc proteins, cell–cell or cell–substrate complex and associated intracellular cytoskeletons, and sarcomere components. These proteins, when mutated, are likely to disrupt force-transmission within a single cardiomyocyte as well as in neighboring cardiomyocytes. However, two newly identified genes, phospholamban and SUR2, a subunit of  $K_{ATP}$  channel, do not fit into this hypothesis. It is highly likely that more genes will be identified as causative factors in DCM after detailed genetic screening. Combination with systemic screening of the known gene mutations in familial DCM patients and their disease phenotype will lead to a better understanding of pathogenesis and prevalence of the monogenic causes of DCM.

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# II

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## GENE TRANSFER FOR COMBATING CARDIOVASCULAR DISEASE

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# 8

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## Gene Therapy vs Pharmacotherapy

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*Ryuichi Morishita, MD, PhD*

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### SUMMARY

Recent progress in molecular and cellular biology has developed numerous effective cardiovascular drugs. However, there are still number of diseases for which no known effective therapy exists, such as peripheral arterial disease, ischemic heart disease, restenosis after angioplasty, vascular bypass graft occlusion, and transplant coronary vasculopathy. Currently, gene therapy is emerging as a potential strategy for the treatment of cardiovascular disease to treat such diseases despite this limitation. The first human trial in cardiovascular disease started in 1994 to treat peripheral vascular disease, using vascular endothelial growth factor (VEGF). Since then, many different potent angiogenic growth factors have been clinically tested to treat peripheral arterial disease. In addition, therapeutic angiogenesis using *VEGF* gene was applied to treat ischemic heart disease from 1997. The results from these clinical trials appear to exceed expectations. Improvement of clinical symptoms in peripheral arterial disease and ischemic heart disease has been reported. Many different potent angiogenic growth factors have been tested in clinical trials to treat peripheral arterial disease or ischemic heart disease. In addition, another strategy for combating disease processes, to target to transcriptional process, has been tested in a human trial. Transfection of *cis*-element double-stranded (ds) oligodeoxynucleotides (ODN) (decoy) is an especially powerful tool in a new class of antigene strategies for gene therapy. Transfection of ds ODN, corresponding to the *cis* sequence, will result in the attenuation of authentic *cis*-trans interaction, leading to the removal of transactors from the endogenous *cis*-elements with subsequent modulation of gene expression. Genetically modified vein grafts transfected with decoy against E2F, an essential transcription factor in cell cycle progression, revealed apparent long-term potency

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in human patients. This review focuses on the future potential of gene therapy for the treatment of cardiovascular disease.

**Key Words:** *cis*-element decoy; antisense; angiogenesis; VEGF; HGF.

## INTRODUCTION

Somatic gene therapy consists of the introduction of normal genes into the somatic cells of patients to correct an inherited or acquired disorder through the synthesis of specific gene products *in vivo*. In general, there are three methods of gene modification: (1) gene replacement, (2) gene correction, and (3) gene augmentation. Gene augmentation is the most promising technique for the modification of targeted cells in therapy for cardiovascular disease. For this purpose, many *in vivo* gene transfer methods have been developed. *In vivo* gene transfer techniques for cardiovascular applications include: (1) viral gene transfer—retrovirus, adenovirus, adeno-associated virus (AAV) or hemagglutinating virus of Japan (HVJ, or Sendai virus); (2) liposomal gene transfer using cationic liposomes; and (3) naked plasmid DNA transfer. These *in vivo* gene transfer techniques have different advantages and disadvantages. Although current *in vivo* methods for cardiovascular gene transfer are still limited by the lack of efficiency and potential toxicity, recent advances in *in vivo* gene transfer may provide the opportunity to treat cardiovascular diseases, such as peripheral arterial disease by manipulating angiogenic growth factor genes.

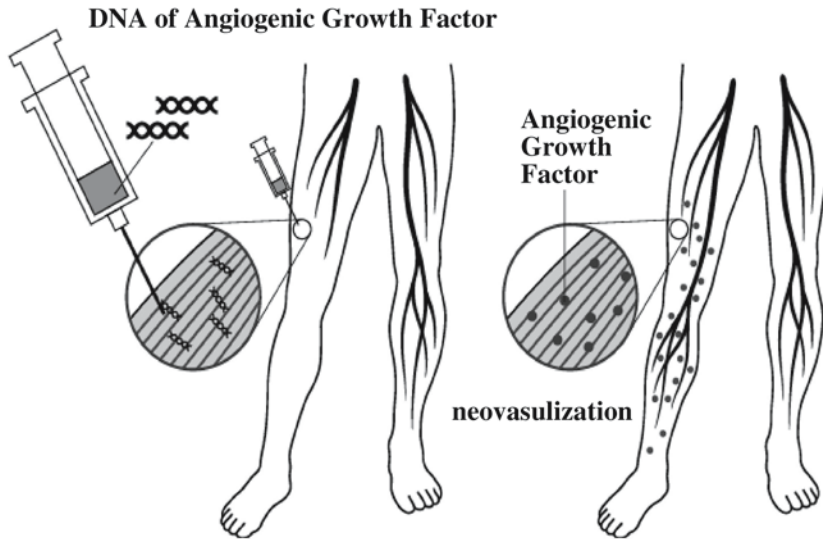
Simple comparison of gene therapy vs pharmacotherapy may not be appropriated. The present promising gene therapy is mainly by locally administrated agents, whereas most of pharmacotherapy is based on oral drug administration. To consider the advantage of gene therapy, one might compare it with recombinant therapy, because both concepts are relatively close. There are at least three advantages of gene therapy. First, it has the potential to maintain an optimally high and local concentration over time. This issue may be critical in the case of arterial gene therapy. In order to avoid side effects in the case of therapeutic angiogenesis, however, it may be preferable to deliver a lower dose over a period of several days or more from an actively expressed transgene in the iliac artery, rather than a single or multiple bolus doses of recombinant protein. Indeed, our initial trial using HGF did not demonstrate the increase in serum hepatocyte growth factor (HGF) level. Second, regarding economics, one must consider which therapy would ultimately cost more to develop, implement, and reimburse, particularly for those indications requiring multiple or even protracted treatment. Third, the feasibility of a clinical trial of recombinant protein is currently limited by the lack of approved or available quantities of human-quality recombinant protein. This may be due, in large part, to the nearly prohibitive cost of scaling up from research grade to human quality recombinant protein. Indeed, the report that compared the effectiveness of fibroblast growth factor-2 (FGF-2) as protein and as naked plasmid DNA, in a porcine model of chronic myocardial ischemia, demonstrated that intramyocardial injection of FGF-2 plasmid was more effective than that of FGF-2 protein in improving regional perfusion and contractility compared to untreated ischemia (1). In contrast, gene therapy also has disadvantages, such as safety aspects, and local and limited effects.

## GENE THERAPY FOR VASCULAR DISEASES

### *Gene Therapy to Treat Peripheral Arterial Disease Using Therapeutic Angiogenesis*

Critical limb ischemia, which is estimated to develop in 500 to 1000 individuals per million per year, is considered one of most suitable diseases for gene therapy. In a large proportion of these patients, the anatomical extent and the distribution of arterial occlusive disease make the patients unsuitable for operative or percutaneous revascularization. Thus, the disease frequently follows an inexorable downhill course. Of importance, there is no optimal medical therapy for critical limb ischemia, as the Consensus Document of the European Working Group on Critical Limb Ischemia concluded. Thus, in patients with critical limb ischemia, amputation, despite its associated morbidity, mortality, and functional implications, is often recommended as a solution to the disabling symptoms, particularly in the case of excruciating ischemic rest pain. Indeed, a second major amputation is required in nearly 10% of such patients. Consequently, the need for alternative treatment strategies in patients with critical limb ischemia is compelling. Therefore, novel therapeutic modalities are needed to treat these patients. In the pathophysiology of the disease, in the presence of obstruction of a major artery, blood flow to the ischemic tissue is often dependent on collateral vessels. When spontaneous development of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, ischemia occurs. Recently, the efficacy of therapeutic angiogenesis using vascular endothelial growth factor (VEGF) gene transfer has been reported in human patients with critical limb ischemia (2–5). Thus, the strategy for therapeutic angiogenesis using angiogenic growth factors should be considered for the treatment of patients with critical limb ischemia. Most of the initial studies have used VEGF, also known as vascular permeability factor, as well as a secreted endothelial cell mitogen. The endothelial cell specificity of VEGF has been considered to be an important advantage for therapeutic angiogenesis, as endothelial cells represent the critical cellular element responsible for new vessel formation (2,3).

A human clinical trial using *VEGF* gene started in 1994 by Professor Isner at Tufts University. An initial trial was performed using a hydrogel catheter with naked *VEGF*<sub>165</sub> plasmid. Although this procedure seems to be effective to stimulate collateral formation in patients with peripheral arterial disease (PAD) (2–5), it is not ideal to treat many patients, as most patients lack an appropriate target vascular lesion for catheter delivery. Thus, his group applied intramuscular injection of naked plasmid encoding *VEGF*<sub>165</sub> gene (Fig. 1). Exceeding expectations, this clinical trial demonstrated clinical efficacy for treatment of PAD (2–5). Since then, numerous angiogenic growth factors, such as VEGF<sub>121</sub>, VEGF-2, and basic-fibroblast growth factor (bFGF) have been tested in clinical trials (6,7). In addition to intramuscular injection of naked plasmid DNA, adenoviral delivery, liposomal delivery of angiogenic growth factors, was also utilized in these trials (2–9), despite an unfortunate accident using adenoviral vector reported at the University of Pennsylvania (10). In addition to the success to the intramuscular injection of VEGF<sub>165</sub> and VEGF2 plasmid DNA, local catheter-mediated *VEGF*<sub>165</sub> gene therapy in ischemic lower-limb arteries after percutaneous transluminal angioplasty (PTA) was also success-



**Fig. 1.** Concept of therapeutic angiogenesis using DNA of angiogenic growth factors.

ful (7). Follow-up digital subtraction angiography revealed increased vascularity in the VEGF-treated groups distally to the gene transfer site and the region of the most clinically severe ischemia (7). A recent report, using adenovirus-encoding VEGF<sub>121</sub>, demonstrated the improvement of endothelial dysfunction in response to acetylcholine or nitroglycerine (8). However, a high incidence of edema has been reported as side effect in the VEGF trial. The recent results from the Regional Angiogenesis with Vascular Endothelial growth factor (RAVE) trial demonstrated that adenoviral VEGF<sub>121</sub> gene transfer was not successful in subjects with intermittent claudication (9). The selection of the agent (VEGF<sub>121</sub> vs <sub>165</sub>), patient population (intermittent claudication vs critical limb ischemia), and outcome measures (peak walking time vs ulcer size) should be considered in the quest for optimal angiogenic strategies that result in the growth of functional blood vessels and improvement in clinical symptoms.

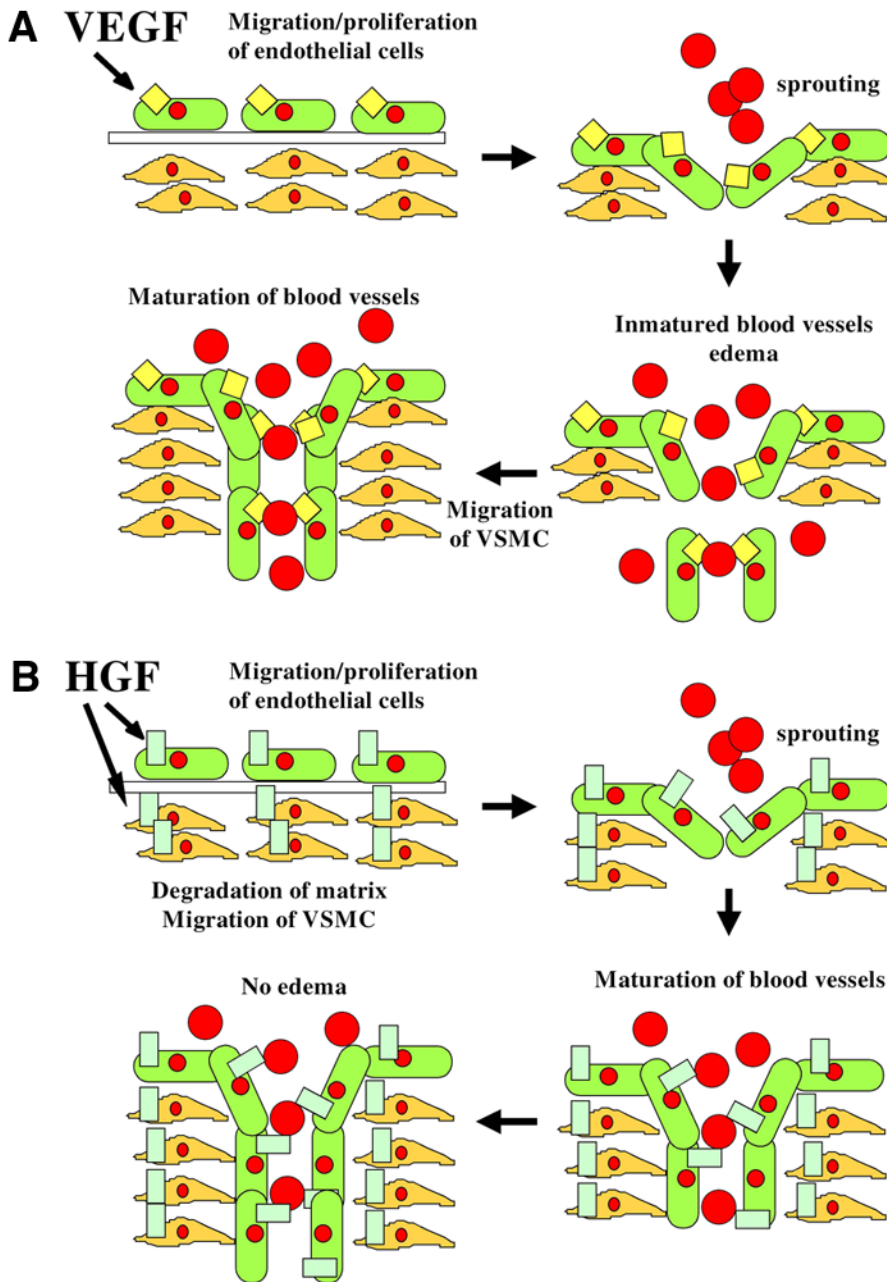
The safety and efficacy of increasing single and repeated doses of intramuscular naked plasmid DNA encoding for *FGF* type 1 administered to patients with unreconstructible end-stage PAD was also reported (6). A significant reduction in pain and aggregate ulcer size was detected after *FGF* gene transfer associated with an increased transcutaneous oxygen pressure and Ankle Pressure Index (ABI) as compared with baseline pretreatment values (6). We also identified HGF as a novel candidate for therapeutic angiogenesis. HGF is a mesenchyme-derived pleiotropic factor that regulates cell growth, cell motility, and morphogenesis of various types of cells, and is thus considered a humoral mediator of epithelial–mesenchymal interactions responsible for morphogenic tissue interactions during embryonic development and organogenesis. We and others previously reported that HGF stimulated angiogenesis in a rabbit ischemic hindlimb model, a rat ischemia model, and mouse ischemia models (11–18). In addition, the angiogenic activity of HGF is more potent than VEGF or bFGF *in vitro* as well as *in vivo* (11, 19). Moreover, transfection of the human *HGF* gene by naked plasmid DNA or HVJ-liposome method resulted in a significant increase in blood flow (14). The angiogenic property of transfection of the *HGF* gene was also proven in a diabetic and a high lipoprotein (a) models (15, 16).

Based upon these findings, we planned a human clinical trial using intramuscular injection of naked human HGF plasmid ( $0.5 \text{ mg} \times 4 \text{ sites}$ ) two times. Currently, HGF gene transfer has been performed in six patients with PAD ( $n = 3$ ) or Buerger disease ( $n = 3$ ) of Fontaine grade III or IV who had failed conventional therapy. Reduction of pain scale (1 cm in visual analog scale) was observed in five of six patients (efficacy rate approx 80%). Increase in ABI to greater than 0.1 was observed in five of five patients (efficacy rate 100%), whereas in one patient we failed to measure ABI before gene therapy because of severe calcification. Importantly, the serum level of human HGF protein did not change during gene therapy. No acute severe complications or allergic events were observed in any patient. Two-month follow-up studies showed no evidence of the development of neoplasm or hemangioma. Although these results are still preliminary, gene therapy using HGF may have therapeutic value to treat PAD. It is noteworthy that there was no evidence of edema in patients that were transfected with the human HGF gene. This finding is in marked contrast to the VEGF trial in which 60% of patients developed moderate or severe edema in a phase I/IIa trial. Currently, there is a phase III trial to treat PAD underway in Japan and a phase II trial in the United States.

We believe that one of the distinguishing features of HGF is that it stimulates the migration of vascular smooth muscle cells (VSMC) without the replication of VSMC, whereas VEGF does not stimulate either the migration or proliferation of VSMC because of the lack of its receptors in VSMC (20). As shown in Fig. 2A, the initial event in angiogenesis induced by VEGF is the migration of endothelial cells, leading to the sprouting of blood vessels. Later, the migration of VSMC occurs as a result of the release of platelet-derived growth factor, followed by the migration of endothelial cells. However, a delay in the maturation of blood vessels might exist in the case of angiogenesis induced by VEGF. In contrast, HGF simultaneously stimulated the migration of both endothelial cells and VSMC (Fig. 2B). Thus, the blood vessels may mature at an earlier time point, thereby avoiding the release of blood-derived cells into the extracellular space, although further studies might be necessary to examine the angiogenic properties among various angiogenic growth factors including HGF, VEGF, and FGF. Although these trials are not complete, the feasibility of gene therapy using angiogenic growth factors to treat peripheral arterial disease seems obtainable in near future. Based upon these properties, it is assumed that the first gene therapy drug may be commercial available in 2005.

### ***Gene Therapy to Treat Myocardial Ischemic Disease Using Therapeutic Angiogenesis***

Similar ideas have been applied to treat coronary artery disease. A human gene therapy trial to treat coronary artery disease using *VEGF*<sub>165</sub> gene has been started by Professor Isner and colleagues (21,22). His group performed intramuscular injection of naked plasmid encoding *VEGF* gene into ischemic myocardium through mini-operation. Similar to human trials in PAD, transfection of *VEGF* gene resulted in a marked increase in blood flow and improved clinical symptoms without apparent toxicity (21). More recently, the results from 13 consecutive patients with chronic stable angina have been reported (22). Although all of them had failed conventional therapy (drugs, percutaneous transluminal coronary angioplasty, and/or coronary artery bypass graft), reduction in the size of the defects documented by serial single-photon emission computed tomography imaging was observed after direct myocardial injection of phVEGF<sub>165</sub> via a minithoraco-tomy (22).



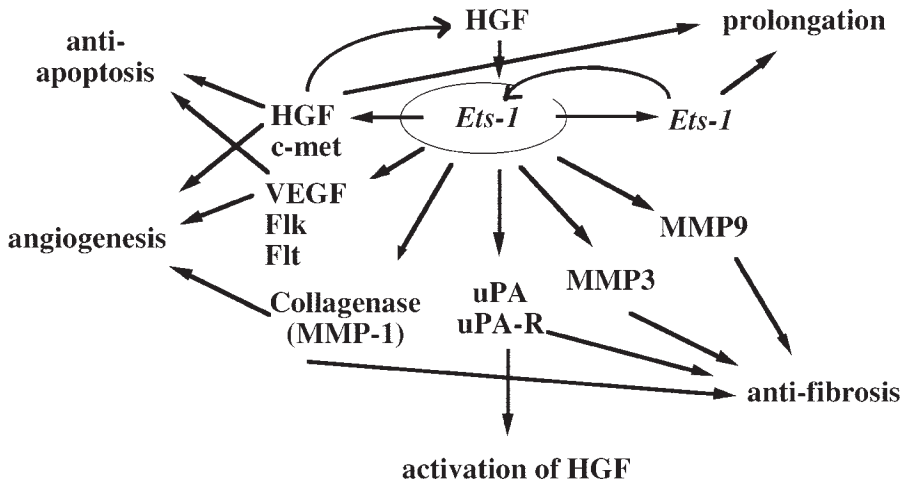
**Fig. 2.** Model of collateral formation induced by vascular endothelial growth factor (VEGF) (A) and hepatocyte growth factor (HGF) (B). HGF stimulated the growth and migration of endothelial cells together with the migration, but not proliferation, of vascular smooth muscle cells (VSMC) through c-met. In contrast, VEGF only stimulated the growth and migration of endothelial cells without the migration or proliferation of VSMC, because of the lack of receptors in VSMC.

These data clearly suggest that *phVEGF<sub>165</sub>* gene therapy may successfully rescue foci of hibernating myocardium. In addition, the recent report summarized the anesthetic management of 30 patients with class 3 or 4 angina, enrolled in a phase 1 clinical trial of direct



myocardial gene transfer of naked DNA-encoding VEGF<sub>165</sub>, as sole therapy for refractory angina. Twenty-nine of 30 patients experienced reduced angina ( $56.2 \pm 4.1$  episodes/week preoperatively vs  $3.8 \pm 1.6$  postoperatively) and reduced sublingual nitroglycerin consumption ( $60.1 \pm 4.4$  tablets/week preoperatively vs  $2.9 \pm 1.1$  postoperatively) (23). Even at 1-yr follow-up, the average number of angina episodes per week and average number of nitroglycerin tablets used per week significantly improved at all measured time points after gene transfer (24). This observation persisted at a 12-mo follow-up, when the average number of anginal episodes was  $10 \pm 19$  and average weekly nitroglycerin tablet usage was  $3 \pm 8$  ( $p < 0.05$  vs baseline for both). Following this success, gene therapy using VEGF<sub>121</sub> gene was performed by intramuscular injection of adenoviral vector (25). A phase I study using adenovirus-mediated transfection of VEGF<sub>121</sub> gene demonstrated clinical safety (25). It is noteworthy that no evidence of systemic or cardiac-related adverse events related to vector administration was observed up to 6 mo after therapy (26). Intracoronary gene transfer of VEGF<sub>165</sub> resulted in a significant increase in myocardial perfusion, although no differences in clinical restenosis rate or minimal lumen diameter were present after the 6-mo follow-up (27). More recently, intracoronary infusion of adenovirus encoding FGF gene was performed in a multicenter trial as phase I/IIa. The report documented that intracoronary infusion of FGF gene improved cardiac dysfunction without severe toxicity (28). Seventy-nine patients with chronic stable angina Canadian Cardiovascular Society class 2 or 3 underwent double-blind randomization (1:3) to placebo ( $n = 19$ ) or Ad5-FGF4 ( $n = 60$ ). Excitingly, a protocol-specified, subgroup analysis showed the greatest improvement in patients with baseline exercise tolerance test  $\leq 10$  min ( $1.6$  vs  $0.6$  min,  $p = 0.01$ ,  $n = 50$ ). In addition, the report documented that treatment of 52 patients with stable angina and reversible ischemia with FGF4 adenoviral injection resulted in a significant reduction of ischemic defect size (29). Currently, the phase IIb/III trials using adenoviral delivery of FGF4 are now underway.

In addition to these angiogenic growth factors, overexpression of HGF was also reported to stimulate angiogenesis and collateral formation in a rat myocardial infarction model (30). Moreover, it was reported that intramuscular injection of HGF gene into the ischemic myocardium resulted in a significant increase in blood flow and prevention of cardiac dysfunction in a canine model (31). The molecular mechanisms of the angiogenic activity of HGF seem to be largely dependent on the ets pathway (an essential transcription factor for angiogenesis), because members of the ets family play important roles in regulating gene expression in response to the multiple developmental and mitogenic signals. The ets family of transcription factors has a DNA-binding domain in common that binds to a core GGA (A/T) DNA sequence. *In situ* hybridization studies have revealed that the proto-oncogene c-ets 1 is expressed in endothelial cells at the start of blood vessel formation, under normal and pathological conditions. Thus, the ets family may activate the transcription of genes encoding collagenase 1, stromelysin 1, and urokinase plasminogen activator, which are proteases involved in extracellular matrix degradation. It is believed that the ets family takes part in regulating angiogenesis by controlling the transcription of these genes, whose activity is necessary for the migration of endothelial cells from pre-existing capillaries. Our previous study demonstrated that HGF upregulated ets activity and ets-1 protein in a myocardial infarction model (30). In addition, exogenously expressed HGF also stimulated endogenous HGF expression through induction of ets activity (32) (Fig. 3), because the promoter region of the HGF gene contains a number of putative regulatory elements, such as a B cell- and a macrophage-specific transcription factor binding site (PU.1/ETS),



**Fig. 3.** Molecular mechanisms of angiogenesis induced by hepatocyte growth factor (HGF) through *ets-1*. HGF stimulated various actions on collateral formation through *ets-1*, revealing that HGF plays a pivotal role as a master gene in the cascade of angiogenesis.

as well as an interleukin (IL)-6 response element (IL-6 RE), a transforming growth factor (TGF)- $\beta$  inhibitory element, and a cAMP response element (33). Severe ischemic heart disease may also be curable using therapeutic angiogenesis by HGF, as a result of the autoinduction of the endogenous HGF system.

Recently, an antifibrotic action of HGF has been identified, as HGF inhibited collagen synthesis through TGF- $\beta$  and stimulated collagen degradation through upregulation of matrix metalloproteinase (MMP-1) and urinary plasminogen activator (uPA) (34). Although the mechanisms through which HGF inhibited TGF- $\beta$  synthesis are not clear, HGF stimulated various metallo-proteases, such as MMP-1 through induction of *ets-1* activity (35). Prevention of fibrosis by HGF was confirmed by previous studies in which administration of human rHGF or gene transfer of human HGF prevented and/or regressed fibrosis in liver and pulmonary injury models (36,37). Thus, HGF may also provide a new therapeutic strategy to treat fibrotic cardiovascular disease, e.g., cardiomyopathy. Our group has also applied to start a human gene therapy protocol using intracardiac muscular injection of HGF plasmid DNA through surgical operation. Overall, the treatment for coronary artery disease may also be curable using therapeutic angiogenesis by gene therapy.

### ***Gene Therapy Into the Brain: A New Era for Cardiovascular Gene Therapy***

In addition, gene therapy may be used to treat cerebrovascular disease. Cerebral occlusive disease caused by atherosclerosis of the cerebral arteries or Moyamoya disease often causes chronic hypoperfusion of the brain. Such a condition leads to not only cerebral ischemic events, but also neuropathological changes including dementia. Currently, an effective treatment to improve hypoperfusion has not yet been established. It is known that ischemic stroke induces active angiogenesis, particularly in the ischemic penumbra, which correlates with longer survival in humans. However, the natural course of angiogenesis is not sufficient to compensate for the hypoperfusion state. In the pathophysiology of the disease, in the presence of the obstruction of a major artery, blood flow to the ischemic tissue is often dependent on collateral vessels. When spontaneous devel-

opment of collateral vessels is insufficient to allow normal perfusion of the tissue at risk, residual ischemia occurs. From this viewpoint, therapeutic angiogenesis must be an effective therapy for cerebral ischemia, resulting in the prevention of future stroke. Angiogenesis can be promoted in the rat brain using adenoviral vectors containing cDNA from bFGF (38). After intraventricular administration of the viral vector, bFGF gene transfer induced angiogenesis in normal rat brain accompanied by an extremely high concentration of bFGF in the brain. In addition to bFGF and VEGF, HGF might be useful to treat ischemic cerebrovascular disease. Our preliminary data revealed that transfection of the HGF gene into the subarachnoid space immediately after occlusion of the bilateral carotid arteries induced angiogenesis on the brain surface and had a significant protective effect against the impairment of cerebral blood flow by carotid occlusion. Moreover, HGF has been the center of interest in neuroprotective substances, because HGF is both a chemoattractant and a survival factor for embryonic motor neurons (39). Sensory and sympathetic neurons and their precursors also respond to HGF with increased differentiation, survival, and axonal outgrowth (39). The broad spectrum of HGF activities suggests that the major role of HGF is to potentiate the response of developing neurons to specific signals. Our recent study demonstrated that gene transfer of HGF into the subarachnoid space has a profound neuroprotective effect against postischemic delayed neuronal death in the hippocampus (40). Stimulation of new vessel formation and prevention of neuronal death by HGF is likely to create new therapeutic options in angiogenesis-dependent conditions, such as stroke, Moyamoya disease, and dementia, although a number of important issues, such as safety and side effects, have not yet been addressed. Our recent report demonstrated that gene transfer into the brain by injection of human *HGF* gene with HVJ-envelope vector into the cerebrospinal fluid via the cisterna magna resulted in a significant decrease in the infarcted brain area without cerebral edema or destruction of the blood–brain barrier (41). Reduction of brain injury by HGF may provide a new therapeutic option to treat cerebrovascular disease.

### ***Gene Therapy for Restenosis After Angioplasty***

Another important cardiovascular disease potentially amenable to gene therapy is restenosis after angioplasty, because the long-term effectiveness of this procedure is limited by the development of restenosis in over 40% of patients. Balloon angioplasty is one of the major therapeutic approaches to coronary artery stenosis. However, restenosis occurs in 30–40% of patients after angioplasty. Intimal hyperplasia develops in large part as a result of VSMC proliferation and migration induced by a complex interaction of multiple growth factors that are activated by vascular “injury.” The process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes that results in mitosis. Therefore, inhibition of the cell cycle using nonphosphorylated retinoblastoma (*Rb*) gene or antioncogenes, such as *p53* and *p21* has been reported in several animal models (42–45). Recently, overexpression of inducible nitric oxide synthase gene has been tested in human subjects, although the results are not yet published.

Alternatively, it has been hypothesized that rapid regeneration of endothelial cells without replication of VSMC may also modulate vascular growth, because multiple antiproliferative endothelium-derived substances (PGI<sub>2</sub>, NO, CNP) are secreted from endothelial cells. This concept was first tested by overexpression of *VEGF*<sub>165</sub> gene (46). Asahara et al. reported a significant inhibition of neointimal formation by acceleration of endothelial cells replication by *VEGF* gene transfer (46). Based on this finding, a

human trial using *VEGF*<sub>165</sub> gene by hydrogel catheter delivery of naked *VEGF*<sub>165</sub> plasmid DNA has been started for restenosis after angioplasty in a peripheral artery (47). Although the final results have not yet been reported, the preliminary results documented the successful inhibition of restenosis after angioplasty (48). A similar trial using *VEGF*<sub>165</sub> gene has been started in Finland. In this trial, *VEGF* gene was transfected by cationic liposome or adenovirus with a catheter into the coronary artery (49). A recent report demonstrated the clinical safety of *VEGF* gene transfer with cationic liposome or adenovirus (49). Although gene transfer with *VEGF* using adenovirus during PTCA and stenting shows that intracoronary gene transfer can be performed safely (no major gene transfer-related adverse effects were detected), no differences in clinical restenosis rate or minimal lumen diameter were present after the 6-mo follow-up (27). Nevertheless, a significant increase was detected in myocardial perfusion in the *VEGF*-treated patients (27). Further studies are necessary to prove the efficacy of re-endothelialization strategy to treat restenosis. In addition, we also reported preclinical experiments in which overexpression of *HGF* gene in balloon-injured arteries could accelerate re-endothelialization, thereby attenuating intimal hyperplasia (50). In this study, we also found that re-endothelialized balloon-injured arteries showed impairment of endothelial dysfunction (50). Further studies are necessary to clarify the utility of gene therapy to treat restenosis after angioplasty.

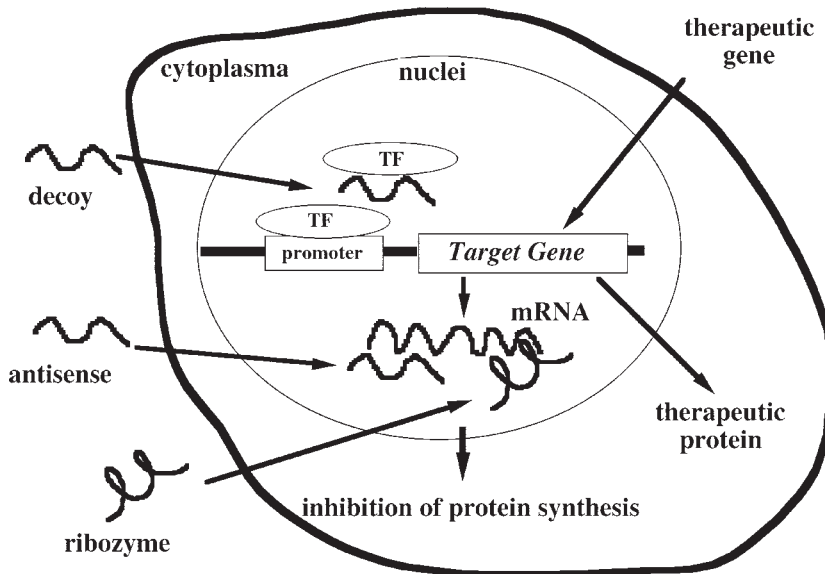
## GENE THERAPY FOR CARDIOVASCULAR DISEASE USING OLIGONUCLEOTIDE-BASED STRATEGY

### *General Concept*

Recent progress in molecular biology has provided new techniques to inhibit target gene expression. The application of DNA technology, such as antisense strategy to regulate the transcription of disease-related genes *in vivo* has especially important therapeutic potential. Antisense oligodeoxynucleotides (ODN) are widely used as inhibitors of specific gene expression, because they offer the exciting possibility of blocking the expression of a particular gene without any change in function of other genes (*see* Fig. 4). Therefore, antisense ODN are useful tools in the study of gene function and may be potential therapeutic agents. The second approach is the use of ribozymes, a unique class of RNA molecules that not only store information but also process catalytic activity. Ribozymes are known to catalytically cleave specific target RNA, leading to degradation, whereas antisense ODN inhibit translation by binding to mRNA sequences on a stoichiometric basis. Theoretically, ribozymes are more effective to inhibit target gene expression. Conversely, we recently have found a novel molecular strategy in which synthetic double-stranded DNA with high affinity for a target transcription factor may be introduced into target cells as a “decoy” *cis* element to bind the transcription factor and alter gene transcription (51).

### *Antisense or Ribozyme-Based Gene Therapy*

As discussed earlier, angioplasty is limited by the development of restenosis in over 40% of patients. Intimal hyperplasia develops in large part as a result of VSMC proliferation and migration induced by the complex interaction of multiple growth factors. First, the effectiveness of antisense ODN against a proto-oncogene, *c-myc*, was reported for the treatment of restenosis (52). Accordingly, inhibition of other proto-oncogenes,



**Fig. 4.** Target sites for antisense, ribozyme and decoy strategies. Antisense, antisense ODN; ribozyme, ribozyme ON; decoy, decoy ODN; TF, transcription factor.

such as *c-myc* by antisense ODN, was also reported to inhibit neointimal formation in several animal models (53). Currently, a phase II trial using antisense *c-myc* to treat restenosis is underway, although its results have not been reported. However, as this trial utilized intracoronary infusion of antisense *c-myc* ODN, several issues, such as low transfection efficiency may limit the efficacy of this strategy.

On the other hand, the process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes that results in mitosis. Our previous data revealed that a single administration of antisense ODN against proliferating cell nuclear antigen (PCNA) and *cdc 2* kinase genes inhibited neointimal formation after angioplasty for up to 8 wk after transfection (54). In addition, a single administration of cyclin B<sub>1</sub>/*cdc 2* antisense ODN combination significantly inhibited the extent of neointimal formation for a period of 8 wk after transfection (55,56). Similar trials have been employed for other vascular diseases, e.g., restenosis after vein grafting and vasculopathy in transplanted heart. This proliferative vascular disease may also be an ideal target for antisense ODN-based strategy. Mann et al. reported that transfection of antisense ODN against proliferating cell nuclear antigen (PCNA) and *cdc 2* kinase resulted in the inhibition of hyperplasia at 2 wk after transfection in a vein graft model (52). Moreover, the prevention of neointimal formation in the balloon injury model by the cell cycle inhibition strategy was sustained long-term over the period of antisense survival (54–57). This may relate to the vascular remodeling induced by the inhibition of cell cycle progression. Indeed, administration of antisense PCNA and *cdc 2* kinase ODN into a vein graft model improved the resistance to diet-induced atherogenesis (58). In addition to the prevention of restenosis after vein grafting, the inhibition of hyperplasia in transplanted hearts has also been reported by Suzuki et al. Transfection of antisense *cdk2* kinase ODN resulted in significant inhibition of VSMC growth in the transplanted heart (59). The first

antisense drug appeared on the market in the United States at the end of 1999 as a novel drug to treat cytomegaloviral-mediated retinopathy.

Another strategy for combating disease processes by targeting to the transcriptional process is the use of ribozymes. We demonstrated the utility of ribozyme oligonucleotides against TGF- $\beta$  by targeting the common sequence of TGF- $\beta$  genes among humans, rats, and mice to treat restenosis in a balloon injury carotid artery model (60). In addition, we also reported inhibition of production of lipoprotein (a) (Lp [a]), which is a risk factor for atherosclerosis, restenosis after angioplasty, cardiac disease, and stroke, without affecting plasminogen level using a ribozyme strategy (61). Nevertheless, similar to antisense strategy, application of ribozyme technology to human gene therapy may require enhancement of the efficiency of cellular uptake and the stability of ribozyme oligonucleotides, because ribozyme oligonucleotides are easily degraded by nucleases because of their RNA backbone.

### *Decoy-Based Gene Therapy*

Transfection of *cis*-element ds ODN (decoy) has been reported as a powerful tool in a new class of antigene strategies for gene therapy (49). Transfection of ds ODN corresponding to the *cis* sequence will result in the attenuation of authentic *cis*-trans interaction, leading to the removal of transactors from the endogenous *cis*-element, with subsequent modulation of gene expression. Therefore, the decoy approach may also enable us to treat diseases by modulation of endogenous transcriptional regulation. Recently, several studies have demonstrated application of the “decoy” ODN strategy as in vivo gene therapy (62–64), and provide evidence of in vivo application of this novel molecular approach as a therapeutic strategy against cardiovascular disease. Many researchers have employed antisense technology as a “loss-of-function” approach at the transcriptional and translational levels, whereas the *cis*-element decoy strategy is also applicable as a “loss-of-function” approach at the pretranscriptional and transcriptional levels to study transcription factors.

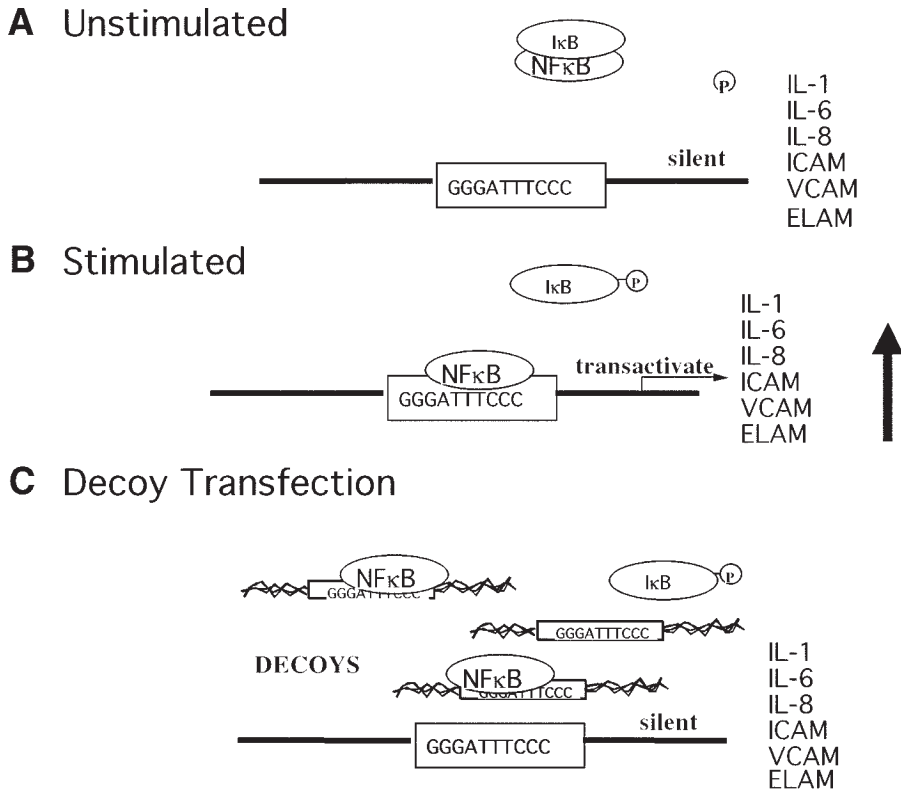
As discussed above, the process of VSMC proliferation is dependent on the coordinated activation of a series of cell cycle regulatory genes, which results in mitosis. A critical element of cell-cycle progression regulation involves the complex formed by E2F, cyclin A, and cdk 2. The dissociation of the transcription factor E2F from the Rb gene product is proposed to play a pivotal role in the regulation of cell proliferation by inducing coordinated transactivation of genes involved in cell-cycle regulation including *c-myc*, *c-myb*, *cdc 2*, PCNA, and thymidine kinase. Accordingly, we hypothesized that transfection of VSMC with a sufficient quantity of decoy ODN containing the E2F *cis* element (consensus sequence “TTTTTCGGCGC”) would effectively bind E2F, prevent it from transactivating the gene expression of essential cell cycle regulatory proteins and thereby inhibit VSMC proliferation and neointimal formation. Transfection of E2F decoy ODN into rat balloon-injured carotid arteries resulted in almost complete inhibition of neointimal formation at 2 wk after balloon injury, accompanied by a reduction in mRNA of PCNA and *cdc 2* kinase, but not  $\beta$ -actin (62). Of importance, sustained inhibition of neointimal formation by a single administration of E2F decoy ODN was observed for at least 8 wk after treatment. Inhibition of neointimal formation by E2F decoy with hydrogel catheter delivery was also demonstrated using a porcine coronary artery model (65). Modification of the delivery of ODN by a hydrogel catheter may overcome issues, such as the low transfection efficiency observed with coronary infusion. Based on these results, we started a clinical trial using hydrogel catheter delivery of E2F decoy to treat

restenosis after angioplasty in April 2000. As of March 2002, we have treated five patients with E2F decoy ODN. We did not observe any side effects up to 6 mo, although the clinical outcome has not yet been evaluated.

In addition, in 1996, clinical application of “decoy” against E2F by Dr. Dzau at Harvard University was also approved by the FDA to treat neointimal hyperplasia in vein bypass grafts, which results in failure in up to 50% of grafts within a period of 10 yr. A proof-of-concept study, the Project in Ex-Vivo Vein Graft Engineering Via Transfection (PREVENT I) study, was the first clinical trial using genetic engineering techniques to inhibit cell-cycle activation in vein grafts (66). This prospective, randomized, controlled trial demonstrated the safety and biological efficacy of intraoperative transfection of human bypass vein grafts with E2F decoy oligonucleotides in a high-risk human patient population with peripheral arterial occlusion. They demonstrated successful inhibition of graft occlusion, accompanied by selective inhibition of PCNA and c-myc expression (66). More recently, similar results were obtained in PREVENT II, a randomized, double-blind, placebo-controlled trial investigating the safety and feasibility of E2F decoy oligonucleotides in preventing autologous vein graft failure after coronary artery bypass surgery (67). The interim results confirmed the safety and feasibility of using this product. Analysis of the secondary end points using quantitative coronary angiography and three-dimensional intravascular ultrasound demonstrated increased patency and positive vascular remodeling (inhibition of neointimal size and volume) in the treated group at 12 mo. Patients examined at follow-up were found to have, on average, a 40% reduction in critical stenosis. Further assessment of this encouraging therapeutic approach should be completed by 2005 in adequately powered phase III studies in coronary and peripheral vessel disease, to determine definitively the extent and duration of clinical benefit. Because E2F has been postulated to play an important role in the pathogenesis of numerous diseases, e.g., vasculopathy after transplantation (68), the development of the E2F decoy strategy may provide a useful therapeutic tool for treating these proliferative diseases.

On the other hand, the transcription factor NF $\kappa$ B also plays a pivotal role in the coordinated transactivation of cytokine and adhesion molecule genes whose activation has been postulated to be involved in numerous diseases, such as myocardial infarction. These diseases are, importantly, potentially amenable to ODN-based gene therapy, because treatment of these diseases is extremely difficult because of the lack of effective pharmacological agents. The pathophysiology of myocardial infarction is quite complicated. Numerous cytokines including interleukin-1, -2, -6, -8, and TNF- $\alpha$ , to name a few, regulate this process. However, gene regulation of many cytokines is relatively simple, because the transcription factor NF $\kappa$ B has been reported to upregulate these cytokines. Interestingly, adhesion molecules such as VCAM and ICAM are also known to be upregulated by NF $\kappa$ B. Accordingly, we hypothesized that myocardial infarction and glomerulonephritis could be prevented by the blockade of genes regulating cell inflammation—the final common pathway that is induced by NF $\kappa$ B binding (*see* Fig. 5). The necessity to block cytokine and adhesion molecule genes at more than one point to achieve maximum inhibitory effects may be a result of the redundancy and complexity of the interactions of these genes.

Importantly, increased NF $\kappa$ B binding activity has been confirmed in balloon-injured blood vessels (69). Our recent study provided the first evidence of the feasibility of a decoy strategy against NF $\kappa$ B in treating restenosis (69). Transfection of NF $\kappa$ B decoy ODN into balloon-injured carotid artery or porcine coronary artery markedly reduced



**Fig. 5.** Mechanisms of NF $\kappa$ B decoy ODN.

neointimal formation, whereas no difference was observed between scrambled decoy ODN-treated and untransfected blood vessels (69,70). Based on the therapeutic efficacy of this strategy, we obtained permission for a second clinical trial using the decoy strategy to treat restenosis from 2001. In addition, the inhibition of VSMC replication was confirmed by the observation that transfection of NF $\kappa$ B decoy ODN inhibited the progression of vasculopathy in cardiac transplantation models (71,72). Blockade of NF $\kappa$ B is also effective in treating reperfusion myocardial injury (63,64). Transfection of NF $\kappa$ B decoy ODN into rat coronary artery prior to left-ascending artery (LAD) occlusion markedly reduced the area of damaged myocytes 24 h after reperfusion. The therapeutic efficacy of this strategy via intracoronary administration immediately after reperfusion, similar to the clinical situation, was also examined. NF $\kappa$ B decoy ODN reduced the damage of myocytes resulting from reperfusion, in contrast with rats treated with scrambled control decoy or vehicle.

Because NF $\kappa$ B has been postulated to play an important role in the pathogenesis of numerous diseases, e.g., cancer and arthritis, the development of a NF $\kappa$ B decoy strategy may provide a useful therapeutic tool for treating these diseases. Furthermore, modifications of ODN composition to prolong decoy stability *in vivo* and/or development of a delivery system into the cardiovascular organs/tissues will be critical to enhance potential therapeutic efficacy (73,74). Despite these limitations, development of this technology offers great promise as a new tool for defining biological processes and treating



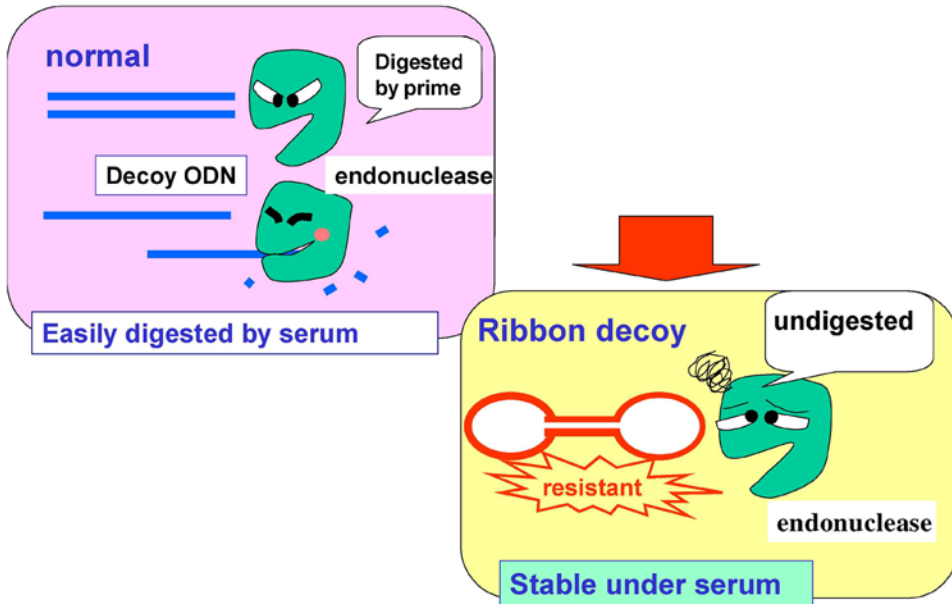
pathological conditions. Overall, this approach is particularly attractive for several reasons: (1) the potential drug targets (transcription factors) are plentiful and readily identifiable; (2) the synthesis of sequence-specific decoys is relatively simple and can be targeted to specific tissues; (3) knowledge of the exact molecular structure of the targeted transcription factor is unnecessary; and (4) decoy ODN may be more effective than antisense ODN in blocking constitutively expressed factors as well as multiple transcription factors that bind to the same *cis* element. Thus, the decoy strategy may be useful for treating a broad range of human diseases. In contrast, because an important concern regarding the decoy strategy revolves around the potential inhibition of normal physiological responses, the application of decoy strategy as gene therapy may be limited to treatment of acute conditions, namely transcription factor-driven diseases.

### *Unresolved Issues in ODN-Based Gene Therapy*

ODN-based gene therapy still has many unsolved problems, such as the short half-life, low efficiency of uptake, and degradation by endocytosis and nucleases. Therefore, many groups currently are focusing on modifications of the gel approach using a catheter delivery system. Further modification of ODN pharmacokinetics will facilitate the potential clinical utility of the agents by: (1) allowing a shorter intraluminal incubation time to preserve organ perfusion; (2) prolonging the duration of biological action; and (3) enhancing efficacy, such that the nonspecific effects of high doses of ODN can be avoided. Regarding the ODN-based strategy as gene therapy, one of the major concerns is nonspecific effects, particularly those of phosphorothioate-substituted ODN (75–78). To overcome these issues, carefully controlled experiments must be performed to eliminate the potential nonspecific effects of ODN-mediated therapy. For gene therapy using an ODN-based strategy, the toxicity of phosphorothioate ODN may also be important. Although low-dosage administration does not seem to cause any toxicity, bolus infusions may be dangerous. Higher doses over prolonged periods of time may cause kidney damage, as evidenced by proteinuria and leukocytes in the urine in animals (77). Liver enzymes may also be increased in animals treated with moderate to high doses. Several phosphorothioate ODN have been shown to cause acute hypotensive events in monkeys (79,80), probably as a result of complement activation (81). These effects are transient, if managed appropriately, and relatively uncommon. This toxicity can be avoided by giving intravenous infusions rather than bolus injections. More recently, prolongation of prothrombin, partial thromboplastin, and bleeding times has been reported in monkeys (82). Alternatively, we recently developed new modification of decoy ODN in order to increase their stability against nucleases. Although the chemical modifications of ODN, such as phosphorothioation and methylphosphonation, were employed, problems with these modified ODNs became apparent including insensitivity to RNaseH, lack of sequence specificity, and immune activation, as described previously. To overcome these limitations, covalently modified ODN were developed by enzymatically ligating two identical molecules, thereby preventing their degradation by exonucleases (Fig. 6) (83). In fact, the transfection of novel AP-1 decoy ODN with circular ribbon structure, prior to the balloon injury procedure, prevented neointimal formation in the rat balloon-injured artery more effectively than nonmodified decoy ODN (83).

### PERSPECTIVES IN GENE THERAPY

Gene therapy in the field of cardiovascular disease would be useful for the treatment of many diseases, including PAD, myocardial infarction, restenosis after angioplasty,



**Fig. 6.** Development of ribbon-type decoy ODN that has the potent resistance to endonuclease.

and rejection in heart transplantation. The first federally approved human gene therapy protocol started on September 14, 1990 for adenosine deaminase deficiency patients. Ten years since the commencement of the first trial, over 4000 patients have been treated by gene therapy. The objectives are generally to evaluate: (1) the in vivo efficacy of the gene transfer method; (2) the safety of the gene transfer method, and (3) the possible therapeutic efficacy. Although there are still many unresolved issues in the clinical application of gene therapy, gene therapy for cardiovascular disease now appears to be not far from reality and it is time to take a hard look at practical issues that will determine the real clinical potential. These include: (1) further innovations in gene transfer methods, (2) well-defined disease targets, (3) cell-specific targeting strategies, and (4) effective and safe delivery systems.

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## Molecular Therapeutic Approaches for Myocardial Protection

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### SUMMARY

Heart failure associated with coronary artery disease is a major cause of morbidity and mortality. Recent developments in the understanding of the molecular mechanisms of heart failure have led to the identification of novel therapeutic targets which, combined with the availability of efficient gene delivery vectors, offer the opportunity for the design of gene therapies for protection of the myocardium. Viral and cell-based therapies have been developed to treat polygenic and complex diseases such as myocardial ischemia, hypertension, atherosclerosis, and restenosis. In addition, cell-based therapies may have potential application in neovascularization and regeneration of ischemic and infarcted myocardium. The recent isolation of regeneration-competent endothelial precursor cells from adult bone marrow provides a novel opportunity for repair of the failing heart using autologous cell transplantation. In this chapter we will focus on the latest advances in the field of gene- and cell-based therapies for treatment of heart failure, and their clinical applications.

**Key Words:** Gene therapy; cell-based therapy; myocardial protection.

### INTRODUCTION

Despite significant advances in the clinical management of cardiovascular disease, acute myocardial infarction (MI), and heart failure (HF) resulting from coronary artery disease (CAD), cardiomyopathy and systemic vascular disease remain the prevalent

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causes of premature death across all age and racial groups (1). The epidemiological impact of heart disease imposes a severe physical and financial strain on health delivery systems. The complexity of the pathological processes leading to heart disease and the lack of specific predictive markers has been a major impediment to the development of effective preventive therapies, despite the identification of various risk factors and sensitive risk assessment technologies (2–4). Consequently, the focus has been on the design of “rescue” treatments for overt symptoms of the disease, such as hyperlipidemia, myocardial ischemia, left-ventricular pump failure, and hemodynamic overload (5). Although these therapies have improved the clinical outlook for patients afflicted by MI and HF, morbidity and mortality associated with these diseases remain high, indicating the need for more effective treatments.

The current availability of efficient vector systems, such as adeno-associated virus (AAV) (6,7) and the recent identification of several gene targets associated with heart disease (8,9) offer opportunities for the design of gene therapies for myocardial protection and rescue. The ability of AAV to confer long-term and stable protein expression with a single administration of the therapeutic gene (10–12) renders them ideally suited for delivery of therapeutic genes. In addition, the repair and vascularization of injured myocardium using autologous cell transplantation may be possible with the recent identification and isolation of cardiomyocyte and endothelial progenitor cells from adult bone marrow and peripheral blood (13–16).

In this chapter we review the major advances in gene- and cell-based therapies for heart disease, with emphasis on strategies for protection and rescue of the heart from failure, their clinical feasibility, and a perspective on future developments in the field. We will highlight the breakthroughs, the challenges in making the transition from preclinical studies to clinical application, and the opportunities ahead in this exciting and growing field.

## STRATEGIES AND TOOLS FOR GENETIC MANIPULATION OF THE MYOCARDIUM

### *Strategies for Genetic Manipulation of the Myocardium*

A wide selection of therapeutic strategies, vectors, and delivery methods are available for genetic manipulation of the myocardium with variable degrees of efficiency (6,17–19). The most common gene therapy strategy for the myocardium involves the exogenous delivery and expression of genes whose endogenous activity may either be defective or attenuated as a result of a mutation or a pathological process. Such “gain-of-function” gene transfer strategies have been widely used with a variety of therapeutic genes, including proangiogenic and survival factors (21,22), antioxidant enzymes (22), and anti-inflammatory cytokines (23). On the other hand, strategies have also been devised for inhibition of genes that may be involved in the development of heart disease. Acute inhibition of transcription and translation can be achieved by treatment with short single-stranded antisense oligodeoxynucleotides, ribozymes, and more recently, using RNA interference technology (12,18,24–26). Inhibition of transcription factor DNA binding using double-stranded “decoy” oligonucleotides for several transcriptional factors has also been employed to inhibit the transactivating activity of target transcription factors (19,27,28). In many instances, the acute inhibition (loss-of-function) of a pathogenic gene is sufficient to prevent the development of disease. For example, the inhibition of



cell-cycle regulatory proteins using decoy oligonucleotides was shown to prevent neointimal hyperplasia and subsequent restenosis following balloon angioplasty or bypass grafting (27).

### *Selection of Therapeutic Target, Vector, and Delivery Strategy*

The choice of therapeutic target, vector, and delivery strategy is, to a large extent, governed by the pathological features of the disease, the putative role of the target gene(s) in the pathophysiological process, and the timing of intervention (29). The efficiency of gene transfer to the myocardium is highly dependent on the type of vector, route, dosage, volume of delivery of the genetic material, and the ability to transduce dividing or non-dividing cells (30,31). Other vector characteristics, such as the capacity to accommodate the transgene, ability to integrate into the host genome, and immunogenicity should also be taken into consideration. The characteristics of the disease dictate whether transient or long-term transgene expression is warranted. Thus, vector systems capable of integrating into the genome of the host and providing sustained transgene expression should be used for gene transfer in conditions requiring prolonged transgene expression, whereas nonintegrating vectors should be used for conditions requiring transient transgene expression.

With regard to the route of administration, intracoronary delivery of the therapeutic material is favored for global myocardial diseases such as HF and cardiomyopathy. The selectivity of coronary endothelium may, however, restrict the diffusion of some vectors, thus limiting distribution and uptake of the therapeutic transgene. In contrast, localized delivery of the therapeutic material by intramyocardial injection may be the preferred method for regional myocardial diseases, such as coronary ischemia or myocardial infarction. This approach has been used for delivery of angiogenic and cytoprotective genes to ischemic myocardium (32). The major shortcoming of direct injection is the fact that transgene expression is restricted to the area surrounding the site of injection. In some cases, this may require multiple injections to adequately cover the affected area. The overall safety and specificity of gene transfer protocols may be enhanced by incorporating regulatory elements that can direct tissue-specific expression of the transgene in response to underlying pathophysiological cues, such as hypoxia, oxidative stress, or inflammation (33–35). This degree of physiological control of transgene expression would allow spatial and temporal control over gene expression and could avert potential biological and ethical problems associated with nonregulated constitutive transgene expression, such as cytotoxicity and germ cell line transmission (36).

### *Nonviral Vectors*

Gene transfer vectors may be classified under three broad categories—nonviral, viral, and cell-based. Nonviral vectors include naked plasmids, cationic liposome and hybrid formulations, synthetic peptides, and several physical methods (31,37–41). However, myocardial gene transfer efficiency using nonviral vectors is low as a result of rapid degradation of the vector, resulting in transient transgene expression. Nevertheless, there have been reports where naked plasmid-mediated gene transfer into the myocardium led to a sustained therapeutic effect (20,42). The efficiency of plasmid gene transfer can be increased by encapsulating the plasmid in neutral liposomes fused to the viral coat of the Sendai virus (hemagglutinating virus of Japan [HVJ]) (43), but transgene expression with this vector system is transient, rendering it unsuitable for use in chronic heart disease.

Electroporation has been used for transfer of naked DNA into embryonic chick hearts *ex vivo* with moderate efficiency (40), but this protocol is impractical for myocardial gene transfer in humans. Application of nondistending pressure in an enclosed environment has been used to deliver oligonucleotides *ex vivo* to the heart (41) and vein grafts (44), highlighting a potential application of this technique for genetic engineering of blood vessels and other organs in preparation for transplantation. Other nonviral methods of gene transfer, such as cationic liposomes, calcium phosphate, and particle bombardment, have shown limited efficacy in myocardial gene therapy (17). A promising new delivery technology uses synthetic peptide carriers containing a nuclear localization signal to facilitate nuclear uptake of the target cDNA (39). These peptide–DNA heteroplexes are recognized by intracellular receptor proteins and imported into the nucleus, where the target cDNA is transcribed.

### *Viral Vectors*

Recombinant viruses have become the preferred vectors for myocardial gene transfer because they can deliver genetic material into cells with higher efficiency than nonviral vectors (6) and are capable of sustaining expression of the therapeutic gene for extended periods of time. However, in some instances, a robust immune reaction may be triggered by the host in response to the viral proteins synthesized by the vector. This may reduce the efficiency of gene transfer and the sustainability of transgene expression (45). Furthermore, although the viral vectors used in gene therapy are replication-deficient, there is the possibility, albeit remote, that these vectors may revert to replication proficiency, thus raising safety concerns about biological hazards such as oncogenesis and insertional mutagenesis (46).

Adenoviruses are the most widely used viral vectors (45). These viruses can transduce a wide variety of myocardial cell types and can accommodate large DNA inserts. The vector infects both dividing and terminally differentiated cells. However, the cytotoxicity associated with induction of the immune response and the episomal localization of the viral genomes results in rapid loss of transgene expression even in the absence of cell division (45). A new generation of “guttled” adenoviral vectors has been developed in which the host inflammatory response is highly attenuated by removing all of the adenoviral coding sequences (47). These adenoviral vectors can accommodate very large DNA fragments and may be useful for delivering multiple genes.

AAV has emerged as the vector of choice for myocardial gene transfer because of its high myocardial tropism and ability to stably transduce terminally differentiated myocytes with high efficiency (10,11). Intramyocardial delivery of AAV is usually more efficient than intracoronary delivery; however, the efficiency of the latter method can be improved by transient permeabilization of the endothelium with histamine. The vector is poorly immunogenic (48), thus minimizing inflammatory damage. The major limitation of the vector is its inability to accommodate large DNA inserts (transgene size is restricted to 4 Kb or less) (7); however, trans-splicing between two separate AAV vectors has recently been used as a strategy for delivery of genes greater than 4Kb (49).

RNA-based retroviral and lentiviral vectors have not found widespread application in myocardial gene transfer protocols for several biological and technical reasons (50,51). These vectors integrate into the host genome leading to the possibility of long-term transgene expression (50). However, retroviral integration requires cell division, rendering these vectors inefficient in transduction of adult cardiomyocytes. Further-

more, retrovirally delivered transgenes are prone to transcription silencing, which may significantly shorten the duration of transgene expression. Production of high-titer retrovirus preparations is difficult; however, recent improvements in packaging systems, such as the use of pseudotyped viral coats incorporating the vesicular stomatitis virus G protein (VSV-G) has greatly improved the stability of the viral particles and has allowed transduction of a wider spectrum of cell types with relatively high efficiency (51). In contrast with the oncoretroviruses, the human immunodeficiency virus (HIV-1)-related retroviruses can infect both dividing and quiescent cells. Moderate transgene expression has been observed in the heart following transduction with a pseudotyped lentivirus (52,53).

Other viral vector systems currently used for gene transfer, such as herpes simplex viruses (HSV) and alphaviruses, have had limited application in myocardial gene transfer. The ability of HSV-based vectors to accommodate very large DNA fragments provide an advantage for the transfer of very large genes, such as dystrophin or sarcoglycans for treatment of inherited cardiomyopathies (54). Alphaviruses are positive-strand RNA viruses based on the Semliki Forest virus (SFV) and Sendibis virus (55). These viruses have recently been used for very rapid and efficient transduction of several cells and tissues in vitro (56). These viruses are capable of expressing transgenes within 24 h of transduction in the heart with minimal cytotoxicity, suggesting their potential application for gene manipulation in acute myocardial disease such as MI.

### *Cell-Mediated Gene Delivery*

A number of cell types have also been used as vectors for delivery of genetic material to tissues. The recent identification and isolation of endothelial and cardiomyocyte precursor stem cells from adult bone marrow and peripheral blood (13,15) provides a nondepleting, self-renewing autologous cell source that can simultaneously be used as substrate for regeneration and reconstruction of injured myocardium and blood vessels, and as vehicles for delivery of therapeutic genes. For example, the cells could be engineered ex vivo to express cytoprotective and/or proangiogenic genes that would promote survival of the grafted cells and neovascularization of the infarcted myocardium (57). Macrophages, erythrocytes, and vascular endothelial cells have also been successfully transduced ex vivo with retroviral vectors for efficient delivery of therapeutic genes into tissues (58,59). Macrophages genetically engineered to express protective genes under endogenous regulation by hypoxia may have potential application for targeted delivery of genes in myocardial ischemia.

## GENE THERAPY FOR MYOCARDIAL PROTECTION

### *Pathogenesis of Heart Disease and Targets for Gene Therapy*

Myocardial ischemia associated with CAD is the primary cause of myocardial failure (60). Systemic hypertension, disorders of lipid metabolism, and diabetes are the predominant predisposing factors for CAD (2). Acute ischemic events, if sufficiently prolonged, will lead to irreversible damage and infarction, underlined by alterations in membrane fluidity, intracellular hydrogen ion concentration and metabolic activity, and eventual cell death, resulting in arrhythmia and impaired pump function (61). Paradoxically, reoxygenation of the ischemic myocardium induces a robust increase in reactive oxygen species (ROS), which triggers a profound inflammatory response that may exacerbate the

damage initiated during ischemia (61–63). In time, the left ventricle undergoes a process of remodeling characterized by myocyte hypertrophy, interstitial fibrosis, chamber dilatation, and increased propensity for contractile dysfunction that ultimately leads to ventricular failure (64). The remodeling process is complex and highly dependent on the activity of matrix metalloproteinases (MMPs), a group of zinc-dependent proteases that are involved in extracellular matrix degradation (65). Chronic ischemic heart disease is also characterized by heightened inflammatory state and oxidative stress (66,67). The increased levels of proinflammatory cytokines depress myocardial contractility and activate neurohormonal systems such as the renin-angiotensin system, which promote ventricular fibrosis and remodeling.

### *Gene Therapy for Myocardial Ischemia*

The vascular endothelium usually remains in a quiescent, nonproliferative state, and with the exception of the female reproductive tract and neoplastic disease, postnatal neovascularization is rare (68). However, injury, inflammation, and oxidative stress activate the endothelium, resulting in cell proliferation, migration, and formation of new vascular networks by angiogenesis (68). In patients and animal models with ischemic heart disease, the progressive occlusion of the coronary artery leads to a chronic imbalance in myocardial oxygen supply and demand, which stimulates the development of collateral vessels aimed at maintaining tissue perfusion and oxygenation (69). This native adaptive response of the myocardium, however, does not provide adequate compensation in face of severe ischemia, and depression of cardiac function ensues, which in time leads to heart failure.

Evidence of enhanced neovascularization and functional recovery of ischemic myocardium has been reported in several animal and human studies after exogenous supplementation of proangiogenic cytokines by gene transfer (70–76). This novel strategy, commonly known as therapeutic angiogenesis, offers a potentially efficacious method for treatment of coronary artery disease in clinical cases where percutaneous angioplasty or surgical revascularization has been excluded. Proof-of-principle for therapeutic angiogenesis has been demonstrated in several animal models of hindlimb and myocardial ischemia by gene transfer of vascular endothelial growth factor (VEGF) (36,72,73,75,77) and hepatocyte growth factor (HGF) (74,78,79). In all cases, improvement in tissue perfusion was accompanied by morphological and angiographic evidence of new vessel formation, thus establishing a relationship between improved tissue viability and neovascularization. For example, Mack et al. (71) showed that intramyocardial delivery of VEGF<sub>121</sub> by adenovirus led to an improvement in regional myocardial perfusion and left-ventricular function in response to stress in an ameroid constrictor model of chronic myocardial ischemia in pigs. Using intracoronary injection of an adenovirus vector encoding human fibroblast growth factor (FGF)-5, Giordano et al. (72) also showed a significant improvement in blood flow and a reduction in stress-induced functional abnormalities as early as 2 wk after ameroid placement around the proximal left circumflex coronary artery in pigs, in association with an increase in capillary-to-fiber ratios.

Several phase I and II clinical trials of angiogenic gene therapy have been carried out with patients suffering from myocardial and limb ischemia (32,75,80–82). These safety trials, although they consist of small, nonrandomized patient samples, nevertheless demonstrate the potential of angiogenic gene therapy for treatment of ischemic heart disease. Losordo et al. (20) carried out a phase I study in five male patients aged 53–71 yr with

angiographic evidence of CAD who did not respond to conventional antianginal therapy. The authors reported that direct intramyocardial delivery of naked plasmid encoding VEGF<sub>165</sub> into the ischemic myocardium resulted in significant reduction of anginal symptoms and modest improvement in left-ventricular function concomitant with reduced ischemia and improved Rentrop score. Vale and colleagues (80) carried out a randomized, single-blinded placebo-controlled phase I trial in patients with chronic myocardial ischemia using catheter-based delivery of naked VEGF<sub>165</sub> assisted by electromechanical NOGA mapping of the left ventricle. The results of this study indicated significant reductions in weekly anginal attacks for as long as 1 yr after gene delivery in the treated patients, in contrast with the patients receiving placebo. The reduction in anginal episodes was accompanied by improved myocardial perfusion as evidenced by SPECT-sestamibi perfusion scanning and electromechanical mapping. Recently, Grines and colleagues (81) completed the Angiogenic GENE Therapy (AGENT) double-blinded randomized, placebo-controlled trial using dose-escalating adenovirus-mediated intracoronary delivery of FGF-4 in patients with angina, in order to evaluate the safety and efficacy of this protocol in reducing ischemic symptoms. The authors reported increased exercise tolerance and improved stress echocardiograms at 4 and 12 wk after gene transfer in the patients that received FGF-4 gene therapy compared with the patients receiving placebo. The long-term outcome beyond 12 wk, however, has not been followed.

The success of these initial small-scale phase I and phase II trials warrant larger and more adequately controlled later phase trials. However, several issues relating to feasibility, safety, and sustainability require further investigation before therapeutic angiogenesis may be envisaged as a viable therapeutic option for treatment of ischemic heart disease. The broad issue of safety of the approach requires systematic evaluation. This is particularly relevant in light of recent evidence that transplantation of myoblasts constitutively expressing VEGF under a retroviral promoter into mouse hearts led to intramural angiomas followed by heart failure and death (36).

### ***Gene Therapy for Protection From Ischemia and Reperfusion Injury***

The continuum of myocardial injury that is initiated by a coronary ischemic event and perpetuated by reperfusion (I/R injury) may be clinically manifested in patients undergoing thrombolytic therapy following an acute coronary episode. The increase in ROS formation during reperfusion of the ischemic myocardium may eventually deplete the buffering capabilities of endogenous antioxidant systems, thereby exacerbating the cytotoxic effects of these reactive species (83). The development of gene therapies for acute myocardial infarction has been difficult because the time required for transcription and translation of therapeutic genes with the current generation of vectors exceeds the time window for successful intervention. An alternative gene therapy for myocardial protection is to “prevent” I/R by the transfer of cytoprotective genes into the myocardium of high-risk patients prior to ischemia using a gene delivery method that could confer long-term therapeutic gene expression. This novel concept of “preventive” gene therapy would protect the heart from future I/R injury, thereby minimizing the need for acute intervention. Given the prominent role of oxidative stress in I/R injury, a therapeutic approach aimed at increasing endogenous antioxidant reserves should, in principle, be a useful strategy for prevention/protection in patients at risk of acute MI. This strategy would potentiate the native protective response of the myocardium (84), rendering it resistant to future ischemic insults.

We have evaluated the feasibility of antioxidant enzyme gene transfer as a long-term first line of defence against I/R-induced oxidative injury, using an recombinant AAV vector for intramyocardial delivery of heme oxygenase-1 (*HO-1*) gene in a rat model of myocardial I/R injury (22). Our findings show that *HO-1* gene delivery to the left-ventricular risk area several weeks in advance of myocardial infarction results in approx 80% reduction in infarct size. The reduction in myocardial injury in the treated animals is accompanied by decreases in oxidative stress, inflammation, and interstitial fibrosis. Consistent with the histopathology, echocardiographic assessment showed post-infarction recovery of left-ventricular function in the *HO-1* treated animals, whereas the untreated control animals presented evidence of ventricular enlargement and significantly depressed fractional shortening and ejection fraction. Thus, these findings suggest that AAV-mediated delivery of *HO-1* may be a viable therapeutic option for long-term myocardial protection from I/R injury in patients with CAD. Comparable findings were found with extracellular superoxide dismutase (*ecSOD*) gene transfer (85,86). This secreted metalloenzyme plays an essential role in maintenance of redox homeostasis by dismutating the oxygen free radical superoxide. Our findings showed that long-term survival after acute MI is improved in the *ecSOD*-treated animals relative to the animals treated with the control vector, in parallel with smaller infarcts and decreased myocardial inflammation (87). Efficient protection from I/R injury has also been achieved by overexpression of other major antioxidant enzyme systems, such as *ec-SOD* (88), catalase (89), and glutathione peroxidase (90), stress-induced heat shock proteins (HSP), such as HSP 70 (91) and HSP 27 (92), survival genes (*Bcl-2*, *Akt*) (21,93), as well as immunosuppressive cytokines (23), adenosine A<sub>1</sub> and A<sub>3</sub> receptors (94), kallikrein (95), caspase inhibitor (96), and HGF (74).

The inhibition of proinflammatory genes involved in the pathogenesis of I/R injury offers another option for cardioprotection. Morishita et al. (28) showed that pretreatment with a decoy oligonucleotide capable of inhibiting the transactivating activity of the proinflammatory transcription factor NF $\kappa$ B reduces MI after coronary artery ligation in rats. Similarly, intravenous administration of antisense oligonucleotide against angiotensin converting enzyme mRNA (97) or angiotensin AT<sub>1</sub> receptor (94) significantly reduces myocardial dysfunction and injury following ischemia and reperfusion. Although the rapid *in vivo* degradation of oligonucleotides would preclude their use in long-term myocardial protection, they may be useful in treatment of acute myocardial ischemia and cardiac transplantation (98) by providing a tool for inhibiting of prooxidant, proinflammatory and immunomodulatory genes activated by ischemia and reperfusion. For example, treatment with antisense oligonucleotide directed against intercellular adhesion molecule-1 (ICAM-1) was shown to prolong cardiac allograft tolerance and long-term survival when administered *ex vivo* prior to transplantation into the host (99). Such an approach could be beneficial in the preparation of donor hearts for transplantation. For example, oligonucleotide-mediated inhibition of anti-inflammatory genes and adhesion molecules in donor organs in advance of transplantation could be used to suppress the acute inflammatory response that ensues upon reperfusion of the transplanted organ in the recipient.

### ***Gene Therapy for Myocardial Hypertrophy and Remodeling***

The progression of HF resulting from hemodynamic overload, chronic myocardial ischemia, or acute MI is invariably accompanied by hypertrophy and remodelling of the left ventricle (100). This process, which usually begins as an adaptive physiological

mechanism aimed at normalizing wall stress in response to the increased load or myocyte death from infarction, eventually becomes maladaptive, resulting in alteration in ventricular geometry, mechanical decompensation, and contractile failure. Following MI, the left ventricle undergoes an early healing phase during which the infarcted area expands, resulting in wall thinning and ventricular dilation that leads to increased wall stress. This is followed by long-term dilation of the noninfarcted region, and myocyte hypertrophy and interstitial fibrosis leading to ventricular chamber distortion and enlargement (100,101).

Inhibition of ventricular remodelling is a prime target in the treatment of HF, and the long-term survival benefits of therapies such as ACE inhibition and  $\beta$ -blockade in patients suffering from MI or HF are attributed, at least in part, to a decrease in left ventricle remodeling. Pharmacological inhibition of these pathways attenuates the hypertrophic and remodeling process and delay the progression of disease (5). More recently, treatment with MMP inhibitors was shown to effectively attenuate post-infarction LV dilation (102), suggesting that this could be a potential therapeutic strategy for HF. Genetic manipulation of these targets may prove to be an effective alternate therapy to current pharmacological approaches for treatment of HF. Gene therapies aimed at inhibiting hypertrophic and profibrotic pathways should be useful in limiting the extent of remodeling. For example, inhibition of  $AT_1$ -receptor signaling by antisense reduces cardiac hypertrophy in a renin-overexpressing transgenic rat independently of systemic effects (103), suggesting a role of local ANG II in inducing the hypertrophic phenotype. A similar approach could be used for inhibition of cardiostrophic factors, such as calcineurin and protein kinases (104). Antisense inhibition of myocardial transforming growth factor (TGF)- $\beta$ 1 factor signaling and metalloproteinase activity could be employed as strategies to reduce fibrosis and remodeling. Conversely, myocardial overexpression of antihypertrophic factors may be used as a strategy to reverse hypertrophy in failing hearts. Li et al. (105) demonstrated that cardiac specific overexpression of insulin-like growth factor-1 in mice prevented myocyte death in the viable myocardium and attenuated ventricular dilation and hypertrophy after MI. Similarly, cardiac overexpression of glycogen synthase-3 $\beta$ , an endogenous antagonist of calcineurin action, was reported to inhibit hypertrophy in response to chronic  $\beta$ -adrenergic stimulation and pressure overload (106). Overexpression of cyclin-dependent kinase inhibitor p16 has also been shown to reduce cardiac hypertrophy in response to endothelin-1 (ET-1) (107), in agreement with findings that cyclin-dependent kinase inhibitors play an essential role in inhibition of pressure-induced hypertrophy (108). Systemic overexpression of vasodilatory genes such as NOS (109), ANP (110), and kallikrein (111) were effective in reducing cardiac hypertrophy and fibrosis in hypertensive rat models.

### ***Cell-Based Therapy for Myocardial Ischemia***

More recently, cell-based therapy has emerged as an exciting alternative strategy for therapeutic angiogenesis and involves the use of endothelial precursor cells as angiogenic substrate. Several reports have documented the existence of blood-borne endothelial progenitor cells (EPC) originating from a common hemangioblast precursor in adult bone marrow (13,112,113). These endothelial lineage cells have the properties of an endothelial progenitor (CD34<sup>+</sup>, Flk-1<sup>+</sup>) and are recruited to foci of neovascularization, such as ischemic muscle (114) and the myocardium (115), where they differentiate into functional endothelial cells, indicating that they may play a role in postembryonic

vasculogenesis in ischemic tissues. The therapeutic potential of these cells as vehicles for tissue salvage and/or regeneration from ischemia has been demonstrated. Local implantation of autologous bone marrow-derived cells in rat (116) and mouse (117,118) ischemic hindlimb induces angiogenesis and partially restores blood flow and exercise capacity in the ischemic limb. Similarly, transplantation of ex vivo-expanded human EPC into nude rats (114,115) and pigs (119) with myocardial ischemia led to increased capillary density and improved ventricular function. More recently it was reported that the number of circulating EPC increases in patients with acute myocardial infarction (114) and is lower in patients with coronary artery disease (120), indicating that these cells may play an essential role in neovascularization of the myocardium in response to ischemia.

The ability to culture and genetically engineer EPC ex vivo with vectors expressing therapeutic genes suggests that these cells may be ideally suited as a substrate for cell-based gene therapy for neovascularization of ischemic tissues. In this scheme, EPC genetically modified to express angiogenic growth factors could serve as a cell substrate for new vessel growth by vasculogenesis, driven by local proliferation and differentiation of the transplanted cells, and as a source of proangiogenic growth factors for growth of preexisting vessels by sprouting. This concept was recently validated by Iwaguro et al. (57). Using athymic mice with hindlimb ischemia, they showed that transplantation of murine EPC transduced ex vivo with an adenoviral vector expressing VEGF resulted in more efficient neovascularization and blood flow recovery compared to treatment with nontransduced EPC. The improved neovascularization in the animals treated with VEGF-transduced EPC appeared to be, at least in part, the result of enhanced EPC proliferation and adhesion. A potential noninvasive approach for angiogenesis of ischemic myocardium in CAD may involve the mobilization of EPC to the ischemic region using conventional pharmacological therapeutic agents used in treatment of CAD, such as statins. Recently, several groups showed that statin administration increased the number of EPC in patients with stable CAD (1) 20,121, suggesting that the beneficial therapeutic effect of these drugs may be mediated via mobilization of EPC and subsequent neovascularization of ischemic myocardium. In addition, Walter et al. (122) showed that statin therapy accelerates re-endothelization of balloon-injured arterial segments in rats, leading to reduction in neointimal thickening.

### ***Cell-Based Therapy for Myocardial Regeneration***

Despite evidence of myocyte replication in the heart (123), the vast majority of adult cardiomyocytes are terminally differentiated, and are thus unable to divide (124). Consequently, the regenerative capacity of the infarcted myocardium is limited (125). Hypertrophy and, possibly, hyperplasia of the surviving myocytes may provide initial structural and functional compensation, but in time these processes lead to maladaptive remodeling of the ventricle and heart failure (100). Cell transplantation (cellular cardiomyoplasty) may offer a potential alternative for reconstitution of infarcted myocardium and recuperation of cardiac function (126). The basic premise of this approach is that repopulation of the necrotic myocardium with replication-competent cells capable of generating force should rescue contractile function and the structural integrity that is disrupted by MI. Several cell-based regenerative strategies have evolved using a variety of substrates, such as skeletal muscle myoblasts (127), fetal (105) and embryonic (128) cardiomyocytes, and autologous marrow-derived mesenchymal cardiomyocyte progenitors (129). The thera-



peutic efficacy of cellular cardiomyoplasty has, however, been inconsistent, and several technical and safety issues remain to be resolved. The optimal time for grafting after injury, the source and availability of cellular substrate, the delivery method, and the immunetolerance of the host to the grafted cells are important technical and safety considerations. The use of an adult self-regenerating autologous source of progenitor cells with the potential for differentiation into cardiomyocytes that would graft and electrically couple to the native myocardium would appear ideal. Mesenchymal cells from the bone marrow stroma of long bones may provide the best option for cellular cardiomyoplasty using autologous cells (130). These cells are multipotent and have been shown to differentiate into functional cardiomyocytes under specific culture conditions (16,131). MSCs can also be induced to differentiate into cardiomyocytes *in vitro* after treatment with the cytosine analog 5-azacytidine (15,129). The differentiated cells display genetic and biophysical characteristics of fetal ventricular myocytes—namely, the expression of a fetal cardiac gene profile and prolonged action potentials (15), as well as expression of functional adrenergic and muscarinic receptors (130). Administration of mononuclear cell preparations harvested from bone marrow has been reported to improve cardiac function in various models of myocardial injury (16,129,132).

Bone marrow mobilization with cytokines is being investigated as a potential strategy for treatment of acute MI. Treatment with stem cell factor and granulocyte-colony stimulating prior and immediately following infarction led to significant regeneration of infarcted myocardium and improvement in ventricular function, chamber dimensions, and long-term survival in mice (133). These findings suggest that mobilization and “homing” of bone-marrow-derived cardiogenic precursors to sites of injury in the heart may constitute a natural repair mechanism that complements native reparative processes (134,135). The mobilization of cardiogenic precursors could potentially be a therapeutic approach for potentiating this indigenous repair mechanism. The regenerative capability of this cardiac “self-repair” mechanism has, however, recently been questioned by several groups (136,137), who have argued that the number of extracardiac progenitors that are capable of migrating to the heart is too small to induce effective long-term regeneration of the myocardium.

From a clinical perspective, the effectiveness and simplicity of bone marrow mobilization protocols is attractive and may hold therapeutic potential as a noninvasive strategy for treatment of acute MI. However, further work is required to establish the lineage of these precursors, the nature of the migratory and homing signals, the mechanism of transdifferentiation, and the optimal timing for therapeutic intervention. Despite these outstanding issues, one group recently demonstrated the feasibility of using unfractionated bone marrow for treatment of acute MI (138). The authors reported that intracoronary delivery of autologous mononuclear bone marrow cells 6 d after infarction led to a reduction in infarct size and improvement in ventricular function and chamber geometry assessed at 10 wk after transplantation. Although this study illustrates the therapeutic potential of bone marrow stem cell transplantation for treatment of MI in humans, the general consensus of those working in the field is that further characterization of the biology of these cells is required before larger clinical trials are undertaken.

## PERSPECTIVES AND FUTURE DIRECTIONS

Several molecular mechanisms underlying many of the most common cardiovascular diseases have recently been identified. This has led to the development of an array of gene

and cell-based strategies with potential therapeutic value for treatment of these diseases. Some of these strategies have already made the transition from the preclinical phase into clinical trial and are now being considered for use in human patients, whereas several others are currently undergoing safety and feasibility evaluation in early phase trials. Notwithstanding these significant advances, there is still need for further developments in several aspects of cardiovascular gene therapy. Progress in vector and delivery technologies have not kept pace with the identification of novel therapeutic targets. All vectors currently in use for transfer of genetic material do not meet the criteria of the "ideal" vector. Emphasis must be placed on the development of vectors that are amenable to endogenous regulation and with the capability of conferring tissue specificity of transgene expression. Such a degree of spatial and temporal control over transgene expression will enhance the safety of human gene therapy protocols and potentially overcome many of the potential ethical issues that can arise as a result of nonspecific transgene expression, such as germ cell line transmission. Much of this development can be carried out using current vector platforms. Rigorous systematic evaluation of the safety and efficacy of delivery strategies and improvement of delivery devices are also essential prerequisites for human gene therapy protocols.

The optimal genetic therapy for complex diseases, such as CAD and MI may require a combination of cell transplantation and proangiogenic gene therapy for long-term sustenance of the regenerated myocardium. Such potentially synergistic combinatorial approaches have seldom been considered in the design of cardiovascular gene therapy strategies, which have traditionally been developed around a single therapeutic target. Genomic profiling and screening is being employed for molecular phenotyping of patients and will permit the detection of disease-causing polymorphisms and the design of individualized therapies. The convergence of gene transfer technology and genomic technology will facilitate the elucidation of novel genes and may help uncover new roles for previously known genes, thereby leading to the discovery of novel therapeutic targets and approaches for myocardial protection.

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## Gene Transfer and the Cardiovascular System

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### SUMMARY

The cardiovascular application of gene transfer and therapy has three overlapping goals. First, it can be seen as a molecular tool to probe pathways and mechanisms that are difficult to elucidate by other means. Second, it is widely used in preclinical studies and a variety of cardiovascular disease models to find the most efficient and safe clinical applications. Lastly, it is increasingly being used in clinical trial settings, for example to attenuate restenosis and vascular graft failure in coronary and peripheral vascular disease. We provide a critical overview of all three spheres of gene therapy-related strategies in cardiovascular disease research.

**Key Words:** Cardiovascular disease; gene therapy; adenovirus; AAV; targeting; vascular; tropism.

### INTRODUCTION

A wide variety of cardiovascular pathologies are potentially amenable to treatment using gene therapy. Examples include myocardial ischemia, vein graft failure, atherosclerosis, hypercholesterolemia, peripheral ischemia, and hypertension. Gene transfer offers the potential for overexpression of candidate therapeutic genes in these conditions, with the ultimate aim of prevention, improvement, or regression of the condition.

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Gene therapy has two distinct modes of use. In monogenic disorders, in which a single mutated or absent gene has been identified as being causal, the aim of gene therapy would be to insert the corrected form of the gene into the appropriate target cell type to restore normal function. A number of monogenic cardiovascular diseases have been well documented, for example, in conditions such as hypertension (1) or hypercholesterolemia (2). However, the majority of cardiovascular conditions are complex, polygenic disorders, in which environmental factors, such as diet, lifestyle, smoking, exercise, and so on also play a highly important role. In such conditions, elucidating causative genes is difficult. Therefore, the potential for gene therapy is not so much to replace malfunctioning genes in these disorders, but to offer an alternative or adjunct to conventional pharmacological approaches. Thus gene therapy may be aimed at manipulating molecular pathways that have been identified as being crucial in the pathogenesis of a disease—for example, the overexpression of antioxidant genes to combat vascular oxidative stress in hypertension.

Rapid progress has been made in gene therapy research in a number of fields, including cardiovascular disease, to the extent that a number of potential therapies are now undergoing extensive clinical trials. This has led to greater understanding of the requirements for optimal gene transfer, and it has become apparent that an idealized gene delivery system should meet a number of criteria. These include provision of efficient gene delivery, selectivity for the target tissue or cell type, nontoxicity and nonimmunogenicity, in vivo stability, and appropriate regulation of transgene expression in vivo, which may be inducible under pathophysiological conditions. Moreover, the desired vector system should be economical to construct and propagate to high titers, and possess targeting capabilities, thus allowing systemic administration directed at target tissues or show minimal dissemination after local application.

Although it is highly unlikely that any vector will ever meet all of these idealized criteria, a great deal of progress has been made toward attaining many of them. This chapter discusses the current strategies and vectors utilized for gene transfer studies and outlines the techniques used to tailor and specifically target these vectors for cardiovascular use, before describing some key cardiovascular disorders in which gene therapy research is becoming increasingly well characterized.

## MODES OF TRANSGENE DELIVERY

The disparate diseases and conditions potentially amenable to gene therapy results in a number of differing approaches for delivery of gene transfer vectors, each with their own advantages and drawbacks. The main approaches for gene transfer used in current studies—cell-based gene transfer, local in vivo and ex vivo delivery, and systemic in vivo delivery—are outlined in this section.

### *Cell-Based Gene Transfer*

In cell-based gene transfer, a patient's autologous cells are harvested, transduced in vitro with the appropriate vector, and reintroduced. As with other gene transfer methods, this approach offers both advantages and disadvantages. The use of autologous cells reduces the likelihood of immune responses. Furthermore, the in vitro phase of the procedure allows for the generation of a homogeneous population of transduced cells that can easily be assessed for transfection and expression efficiencies. However, the in vitro techniques utilized cause an unavoidable delay resulting from the harvest of the cells, genetic modification, screening, propagation, and reintroduction. Additionally, pheno-

typical changes in cells can occur during culture, the harvest and implantation steps each require individual invasive techniques, and the implantation step may in itself result in damage to the target site. Taken together, these drawbacks may underlie the relative paucity of clinical studies based on this approach. Nonetheless, cell-based gene transfer has been successfully used in a rodent model of vascular injury (3) and a murine model of hypercholesterolemia and atherosclerosis (4). Moreover, a pilot study utilizing cell-based gene transfer of the low-density lipoprotein (LDL) receptor in humans found a significant reduction in total cholesterol levels in individuals who have familial hypercholesterolemia (5). Thus it may be the case that further optimization of the techniques involved in cell-based genetic modification may yet result in more widespread use of this approach as a gene therapy tool.

### *Localized Gene Transfer*

#### **EX VIVO DELIVERY**

Gene transfer to tissue *ex vivo* offers an almost idealized approach to gene therapy, as it allows transduction of the target tissue in an isolated manner, making manipulation and access easier, while also circumventing potential problems caused by systemic leakage of the vector. The ready access of the target tissue makes this approach useful in combination with any of the vectors outlined below in the section titled “Vectors for Cardiovascular Gene Therapy.” Clinical applications of this approach are limited to those in which the target tissue is accessible. It has been predominantly investigated in gene therapy for coronary artery bypass vein graft failure (6–9). During the coronary artery bypass procedure there is a window of opportunity following harvest of the vein that allows for direct transduction *ex vivo*, prior to the grafting of the vein to the aorta. Thus, gene therapy vectors can be applied directly to the graft tissue in a manner that avoids systemic administration, and at a lower total dose. A number of candidate transgenes have shown promise in this area, and are discussed in the section titled “Application of Gene Transfer to Cardiovascular Disease.”

#### **IN VIVO DELIVERY**

The inaccessibility of most target sites for *ex vivo* gene transfer, coupled with a need for refinement of systemic gene delivery approaches, has led to the development of local *in vivo* gene transfer strategies. Such techniques generally rely on the use of modified intravascular delivery catheters or impregnated stents. These are initially introduced at a distal site before being advanced to the target area prior to transgene or vector infusion, or deployment of the stent. A number of specialized devices are now available for this purpose, and have been the subject of review (10). Studies of coronary vessels in both animal models (11) and in humans (12) indicate that, with further refinement, localized gene delivery may hold promise as a means to improve longevity and patency of both stent and bypass approaches to coronary artery disease.

### *Systemic In Vivo Gene Transfer*

Systemic administration of gene transfer vectors *in vivo* offers an idealized approach toward the treatment of a number of cardiovascular conditions. Optimization of this approach would potentially allow infusion of a targeted vector into a peripheral vessel, representing a minimally invasive approach, which may also result in less accessible target tissues (e.g., the pulmonary vasculature) becoming amenable to gene therapy.

However, a key characteristic of systemically infused vectors is their high level of sequestration and destruction by the liver. This has been demonstrated for a number of systems, including adenovirus (13,14), adeno-associated virus (AAV) (15), lentivirus (16), and liposomes (17). This dramatically limits systemic administration of gene therapy vectors for use in cardiovascular disease. A great deal of effort is currently being devoted to modifying the natural tropism of viral vectors to circumvent this problem (*see* Section entitled “Targeted Gene Transfer”). However, even in the absence of vector modification, it is still possible to utilize the liver as a “factory” for transgene production if the gene product is soluble and can be secreted into the bloodstream to exert an effect at an extrahepatic site. It is also possible to utilize the hepatic tropism to direct gene transfer to the liver *per se*, with the aim of modifying lipid metabolism (18).

## VECTORS FOR CARDIOVASCULAR GENE THERAPY

Whereas a variety of diseases are potentially suitable targets for gene therapy, most cell types resist the uptake of foreign DNA. This has led to the development of various vehicles or vectors, each with advantages and disadvantages, in order to facilitate efficient gene transfer (Table 1).

### *Adenovirus*

In cardiovascular research, the adenovirus serotype 5 (Ad5) is the most commonly studied and best characterized of the gene transfer vectors. Deletion of the *E1* and *E3* viral genes has produced a replication deficient system, which generates transgene expression in an episomal manner. Ad5 allows packaging of a relatively large transgene (up to 36kb in helper-dependent adenoviral vectors) and can be propagated to high titers using straightforward methods. Furthermore, adenovirus can infect both dividing and non-dividing cells. The ability to transfect vascular cells, coupled with high efficiency of gene transfer, positions adenovirus as a strong candidate vector for cardiovascular gene therapy. It has been demonstrated that adenovirus can transduce up to 100% of endothelial cells *in vitro* (19–21), however, this is lower *in vivo*, reflecting the high tropism for hepatic tissue of adenoviruses (13). Administration of higher doses of adenoviral vector does not improve the therapeutic potential of this system, but instead leads to toxic effects (22). A further limitation to the use of adenoviral vectors is the relatively transient nature of gene expression. Trans-activation of viral genes can result in “leaky” expression, instigating a cytotoxic T-lymphocyte mediated response against infected cells. However, recent studies using “gutless” vectors (18,23) in which the coding sequences for viral structural proteins have been deleted, suggest that it possible to overcome this problem and produce stable, long-term expression.

### *Adeno-Associated Virus*

In contrast with the nonintegrating adenovirus, AAV can integrate into the host genome at a specific position on chromosome 19 (24). For specific integration to occur, the *rep* protein must be present—integration otherwise occurs semirandomly, with a preference for active genes (25). Chromosomal integration may help to promote long-term transgene expression, a key advantage over other vector systems, such as adenovirus, although recent studies now suggest that virally-mediated, long-term expression mainly occurs from episomal vectors (26,27). AAV is a human parvovirus, which can be stripped of all viral coding sequences, allowing inserts of up to 4.5 kb to be packaged. AAV is a

Table 1  
 Characteristics of Common Gene Transfer Vectors

Technique	Advantages	Disadvantages
Adenovirus	<ul style="list-style-type: none"> <li>Broad tropism</li> <li>Only infects nonreplicating cells</li> <li>Relatively large cloning capacity</li> <li>High levels of transgene expression</li> <li>Transient expression increases safety</li> <li>Can be grown to high titers</li> <li>Amenable to retargeting techniques</li> </ul>	<ul style="list-style-type: none"> <li>Broad tropism</li> <li>Transient expression limits applicability</li> <li>Can invoke immune and inflammatory responses</li> </ul>
Adeno-associated virus	<ul style="list-style-type: none"> <li>Integration into host genome at specific site</li> <li>No immune responses</li> <li>Infects dividing and nondividing cells</li> <li>Can be grown to high titers</li> </ul>	<ul style="list-style-type: none"> <li>Possible removal and dissemination of vector after wild-type adenovirus infection</li> <li>Cloning capacity is limited to 5 kb</li> </ul>
Retrovirus	<ul style="list-style-type: none"> <li>Integration into host genome offers longevity of transgene expression</li> <li>Hold relatively large DNA inserts</li> </ul>	<ul style="list-style-type: none"> <li>Integration can be random</li> <li>Only infect replicating cells</li> <li>Difficult to propagate to high titer</li> <li>Very low efficiency</li> </ul>
Naked DNA	<ul style="list-style-type: none"> <li>Nonpathogenic and easy to construct</li> <li>Can deliver large DNA constructs</li> </ul>	
Liposomes	<ul style="list-style-type: none"> <li>Can hold large inserts</li> <li>Can be used in wide range of cells</li> <li>Nonpathogenic</li> </ul>	<ul style="list-style-type: none"> <li>Low efficiency in comparison to viral gene transfer</li> <li>Nonspecific targeting</li> </ul>

replication-deficient system—lytic infection of host cells requires coinfection with a helper virus, such as adenovirus or herpes simplex virus type I. Greater than 90% of the human population is thought to be infected with AAV, although this infection is asymptomatic in nature. These features—stable and long-term transgene expression and low levels of immunogenicity—position AAV as a promising vector system for cardiovascular gene therapy. Two obvious disadvantages in using AAV as a vector for gene transfer, in comparison with others, such as adenovirus, are the relatively small size of insert (4.5 kb) and the markedly reduced efficiency of transduction of vascular tissue. However, it has been shown that AAV can transduce primary cultures of both endothelial and vascular smooth muscle cells from a number of mammalian species, including human (28). The same study further reported *in vivo* transduction of primate carotid arteries. Moreover, transgene expression in excess of 18 mo has been shown using AAV (29), whereas AAV-mediated gene transfer restored normal heart function and increased life expectancy in a hamster model of congenic cardiomyopathy (30). Delivery of vascular endothelial growth factor (VEGF) using AAV has shown benefit in animal models of ischemia (31). Moreover, clinical trials of AAV-mediated gene therapy for cystic fibrosis are underway (32), while initial studies of AAV-2 mediated gene therapy for hemophilia B in man (33) have so far demonstrated the safety profile of this vector. Although development of this approach in the setting of common cardiovascular diseases in humans lags a little behind, longevity of expression coupled with the ability to deliver potentially therapeutic genes in a low-toxicity manner would suggest that AAV will continue to attract attention as a potential gene therapy vector in this field.

### *Retroviruses*

The retroviruses were the first group of viral vectors to be studied for the purposes of gene transfer, starting in the mid-1980s. Research into this class of vector continues, because of their chromosomal integration (resulting in long-term transgene expression) and high efficiency of gene transfer. However, a number of factors limit the applicability of these vectors. First, they are difficult to grow to sufficiently high titers that are required for systemic *in vivo* administration. Second, chromosomal integration occurs randomly, presenting the possibility of mutational events occurring. The requirement that the host cells be replicating for efficient infection to occur (34) further limits the use of these vectors in vascular tissue, as normal vascular smooth muscle cells divide only once in a lifetime. The main application of retrovirus has been in *ex vivo* transduction of cells prior to reimplantation (3). However, the recent development of leukemia in patients treated for severe combined immunodeficiency disease using retrovirally-mediated transduction of bone marrow cells, through insertion of retroviral DNA into the LMO2 proto-oncogene, led to the halting of clinical trials using this vector (35). Currently, trials using retrovirus are continuing on a case-by-case basis. Ultimately, the future of this vector platform (as with other interventions) will depend on the balance between benefit and risk.

The retrovirus-derived lentivirus, which *can* infect nondividing cells, has shown promise in a number of cardiovascular studies in animal models and has been demonstrated to transfer therapeutic genes into human tissue (36), although clinical trials using this vector have not yet been undertaken. Although concerns exist about the use of HIV-derived lentivirus (37), further development of this vector, e.g., through retargeting strategies to alter tropism (38) and potentially improve biosafety, may lead to its more widespread use in cardiovascular research.

### *Nonviral*

Whereas the focus of much of the current research into gene transfer relies on refinement of virally based vectors, a good deal of progress has also been made utilizing nonviral vectors. Indeed, of ongoing cardiovascular gene therapy trials in 2001, just under half used either naked plasmid DNA or liposomal carriers.

The most simple of the nonviral approaches is the direct injection of naked DNA into the target site, or into a site such as skeletal muscle if the intended target is inaccessible. The latter of these methods requires that the gene product be secreted, non-toxic, and easily transported via the blood stream. A variation of this method utilizes a balloon catheter that has been coated with the plasmid DNA to deliver it to the required vascular site. More recently, it has been shown that ultrasound can dramatically enhance the efficiency of transfection of naked DNA to vascular smooth muscle cells and endothelial cells in culture (39,40), and to rat carotid arteries in vivo (41). Liposomal carriers have also been shown to improve transfection efficiency of plasmid DNA (42); however, such systems are at least 20-fold less efficient than viral vectors, such as adenovirus.

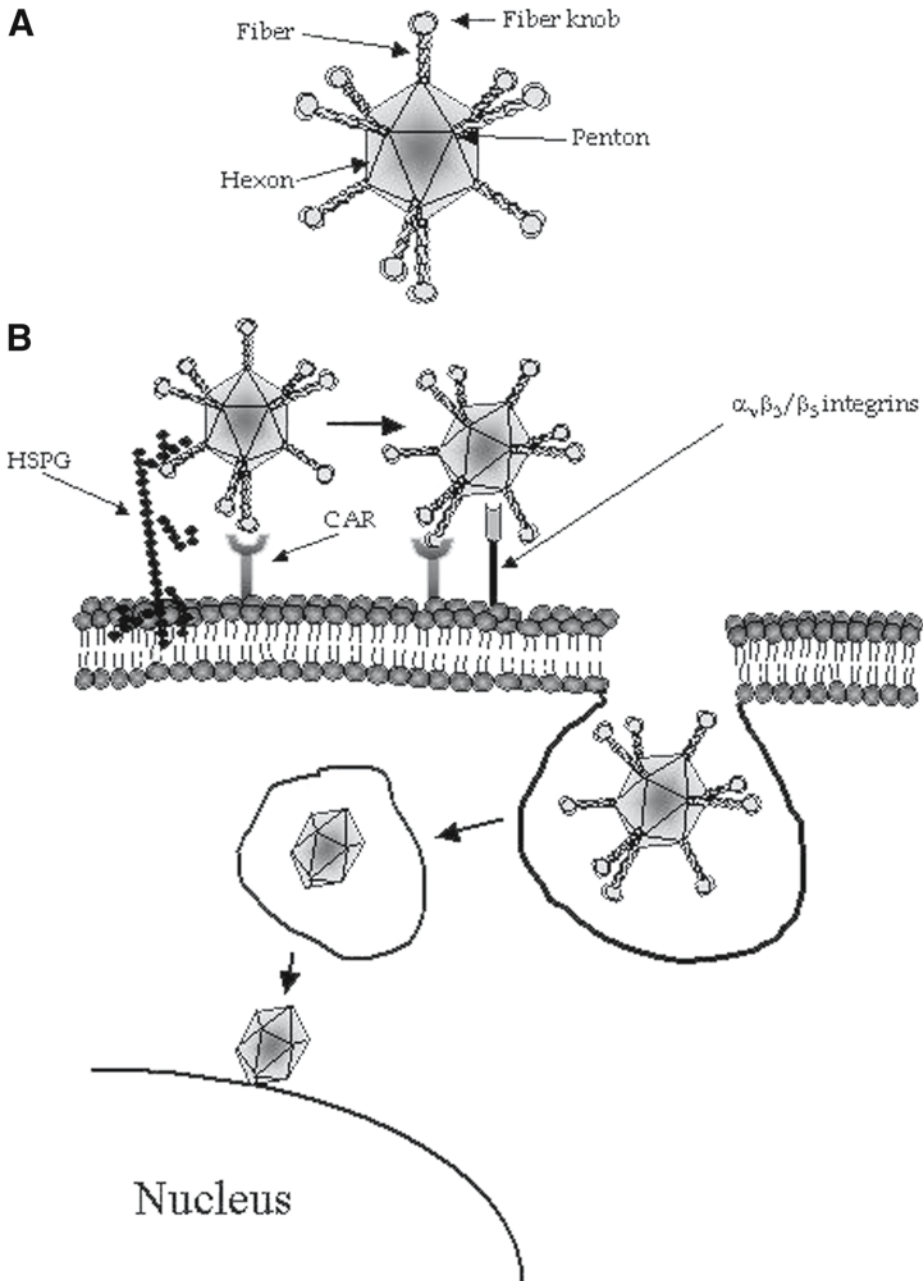
A key advantage of the use of nonviral delivery is the reduction in the likelihood of immune responses or toxic effects, which can be seen after administration of viral vectors. However, the relatively low efficiency of gene transfer, further compounded by a lack of nuclear targeting, represents a significant disadvantage in comparison with viral vectors. Despite such drawbacks, transfer of naked plasmid DNA and liposome-mediated transfer have proved promising in a number of studies, for example, of vascular balloon injury (43) and ischemia (44). In particular, clinical trials utilizing the ex vivo hydrodynamic transfer of decoy oligonucleotides against the cell cycle transcription factor E2F have shown benefit in vein graft occlusion (6), resulting in fewer graft occlusions, revisions, or critical stenoses at a 12-mo time point. Indeed, so promising are the results from these initial trials that E2F decoy is currently being evaluated in two ongoing phase III trials, PREVENT III, and PREVENT IV.

## TARGETED GENE TRANSFER

Although relatively more efficient than other vector systems in terms of vascular gene transfer, the natural tropism for adenovirus has been shown to be the liver and spleen in rodent and primate models (14) and the lung in porcine studies (45) following systemic administration. Thus, there is a need for efficient vascular gene transfer that does not result in high levels of transgene expression in nontarget tissues. Strategies for retargeting of adenoviral, AAV, and other vectors have been developed, forming the basis of a vast body of ongoing research in this field. Optimal targeting of vectors would enable the lowering of the total dose of vector used, thus improving safety profiles, whereas at the same time improving transductional efficiency in the target tissue. The techniques outlined in this section will focus on Ad5 and AAV-2, the most widely characterized systems.

### *Targeting of Adenoviral Vectors*

The process of infection of the Ad5 serotype has been well documented (*see* Fig. 1), and occurs as a two-stage process. In the first phase, attachment to the host cell occurs via an interaction between the knob domain of the capsid fiber and the Coxsackie–adenovirus receptor (CAR) (46,47). Following this, internalization occurs via the activation of  $\alpha_v\beta_3$  or  $\alpha_v\beta_5$  integrins by the penton base of the adenoviral capsid (48,49). Dechecchi et al. showed that Ad5 binding could be inhibited by the presence of heparin, suggesting the



**Fig. 1.** Ad5 structure and infection route. (A) Ad5 has an icosahedral capsid consisting of three main proteins: the fiber, penton base, and hexon, which protects the viral core. (B) Ad5 tethers to the cell surface primarily via interaction of the fiber knob domain with Coxsackie-adenovirus receptor and is then internalized through activation of  $\alpha_v$  integrins by the RGD motif in penton base. Recent data has also demonstrated a role for the Ad5 fiber shaft binding heparin sulphate proteoglycans at the cell surface. The virion then triggers endosomal lysis and trafficks to the nucleus, where it delivers its genome for episomal maintenance. Ad5, adenovirus serotype 5; CAR, Coxsackie-adenovirus receptor; HSPG, heparan sulfate proteoglycans.

presence of a heparan sulphate proteoglycan binding domain (50). More recently, this domain has been defined and localized to the fiber shaft, and its importance in liver transduction demonstrated (51). Thus, strategies aimed at modifying targeting of Ad5 vectors can do so via either ablation or modification of the binding regions within these domains. Such transductional retargeting can be carried out at a genetic or nongenetic level by altering either the coding sequences for structural proteins of the virus or by direct modification of the adenoviral capsid *per se*. Furthermore, retargeting at a transcriptional level via the incorporation of cell-specific promoters to drive transgene expression in target tissues may only further enhance the effects of transductional targeting.

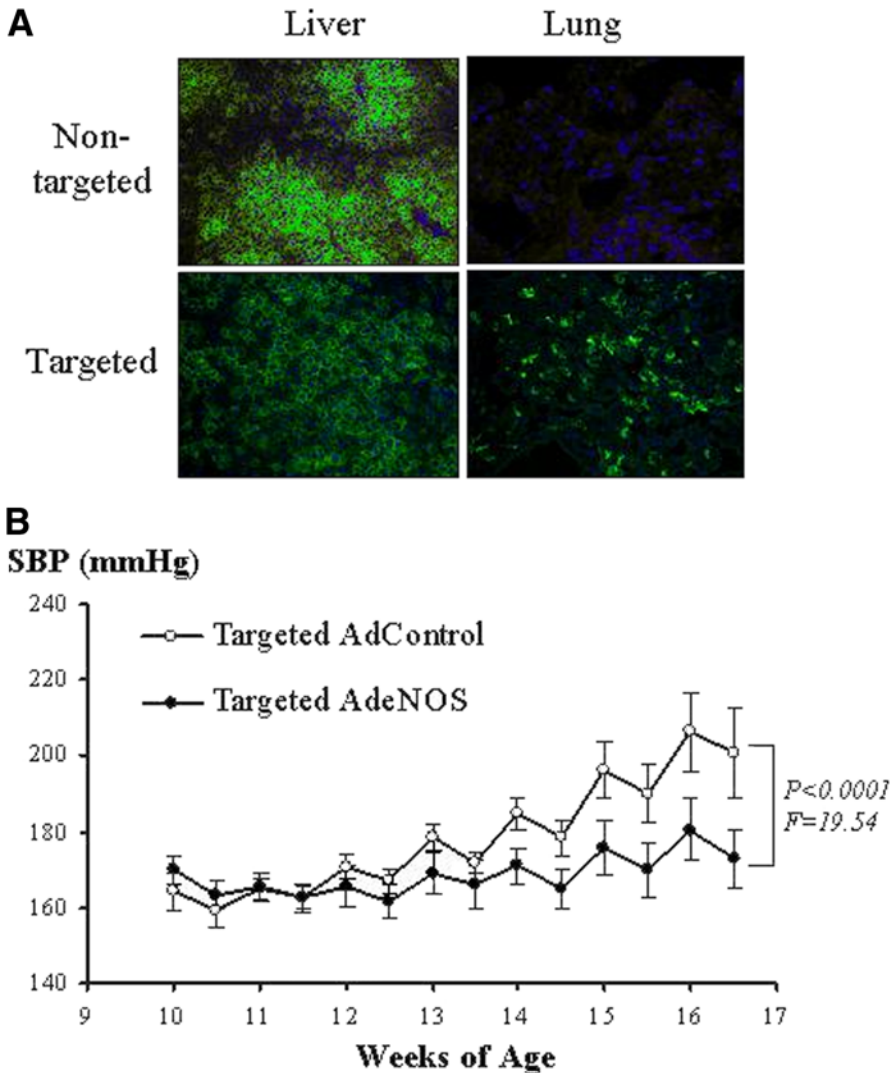
#### **NONGENETIC RETARGETING OF ADENOVIRAL VECTORS**

Nongenetic retargeting usually relies on the use of antigen-binding fragments (Fab), single-chain antibody variable fragments (scFv)/fusion protein constructs, or bispecific antibodies to neutralize the binding domain on the Ad5 knob protein. For example, a bispecific antibody was designed that bound to and neutralized the knob domain of the Ad fiber while simultaneously redirecting it to E-selectin, producing a 300-fold increase in transduction of tumor necrosis factor (TNF)- $\alpha$ -activated porcine endothelium, relative to nontargeted vector (52). Further refinement of this approach has seen the fusion of specific novel targeting peptides (isolated using random phage display [53]) to scFv fragments, resulting in 15-fold increases in transduction of human endothelial cells (54). The same approach has been used to increase transduction of endothelial cells using preexisting markers, such as endoglin (55). The use of bispecific antibodies is potentially extremely powerful. Systemic administration of Ad5 vectors preincubated with a bispecific antibody with affinity for both the Ad5 capsid and angiotensin-converting enzyme (which is expressed at relatively high levels in the pulmonary vasculature) produced a 20-fold increase in transduction levels in rat lung, with an 80% decrease in hepatic transgene expression (56). The effect was further enhanced in subsequent experiments in which this transductional targeting approach was combined with transcriptional targeting, using the endothelial-specific promoter *fms*-like tyrosine kinase (FLT)-1 (57,58). A key set of experiments based on this system by Reynolds et al. resulted in the first study to successfully combine transductional and transcriptional targeting (58; *see also* Fig. 2), one of the most significant demonstrations of detargeting and retargeting approaches. The combination of the endothelial-specific FLT-1 promoter and lung-specific targeting of the adenovirus resulted in a 300,000-fold increase in lung:liver targeting ratio following systemic administration in rats. Whereas this system offers great potential in pulmonary pathologies *per se*, it further acts as striking proof of the concept and potential of dual transductional/transcriptional strategies. These findings suggest that the “holy grail” of a systemically injectable, specifically targeted gene transfer vector may well be tangible, and indeed further study is underway to utilize this system to deliver potentially therapeutic genes (*see* section titled “Hypertension”).

#### **GENETIC RETARGETING OF ADENOVIRAL VECTORS**

A further refinement of transductional retargeting of adenoviral vectors has been the introduction of genetic modification to the capsid. Such single-component systems may offer a number of distinct advantages over the binary systems described previously, in terms of manufacture and quality control. Genetic retargeting strategies are based around the direct insertion of modified ligands (increasingly novel peptide sequences derived from biopanning of random phage libraries), pseudotyping (in which an element of the





**Fig. 2.** Retargeting of adenovirus tropism in vivo. **(A)** Combined transductional and transcriptional targeting of Ad5. Retargeting was carried out by using a bispecific antibody, to direct the vector to the pulmonary vasculature, and FLT-1, an endothelium-specific promoter. Systemic infusion of retargeted reporter vector in rats resulted in a significant shift in the ratio of lung:liver transgene expression, visible as bright immunofluorescence (58). **(B)** Using the same bispecific antibody system, systemic in vivo infusion at 11 wk of age of a targeted AdeNOS vector resulted in a significant and sustained reduction in systolic blood pressure relative to a similarly targeted control vector in the stroke prone spontaneously hypertensive rat (unpublished data from our group). AdeNOS, adenovirus-encoding endothelial nitric oxide synthase; SBP, systolic blood pressure.

viral capsid is replaced by a corresponding element of a different serotype), or genetic ablation of native tropism by disruption of ligand binding motifs. These techniques are often used in varying combinations with each other.

#### PSEUDOTYPING

In pseudotyping, the main approach has been to replace the Ad5 fiber shaft or knob domain with that of another serotype that does not bind to CAR. Studies utilizing this

method have shown improved gene transfer to endothelial cells, vascular smooth muscle cells, and blood vessels *ex vivo* (59–61) using particular pseudotyped Ad5 variants. It has also been noted that interspecies differences exist in transduction levels produced by pseudotyped vectors (62), suggesting differences in biodistribution of receptors for these modified viruses across species and providing a note of caution as to the extrapolation of results from animal studies to human disease.

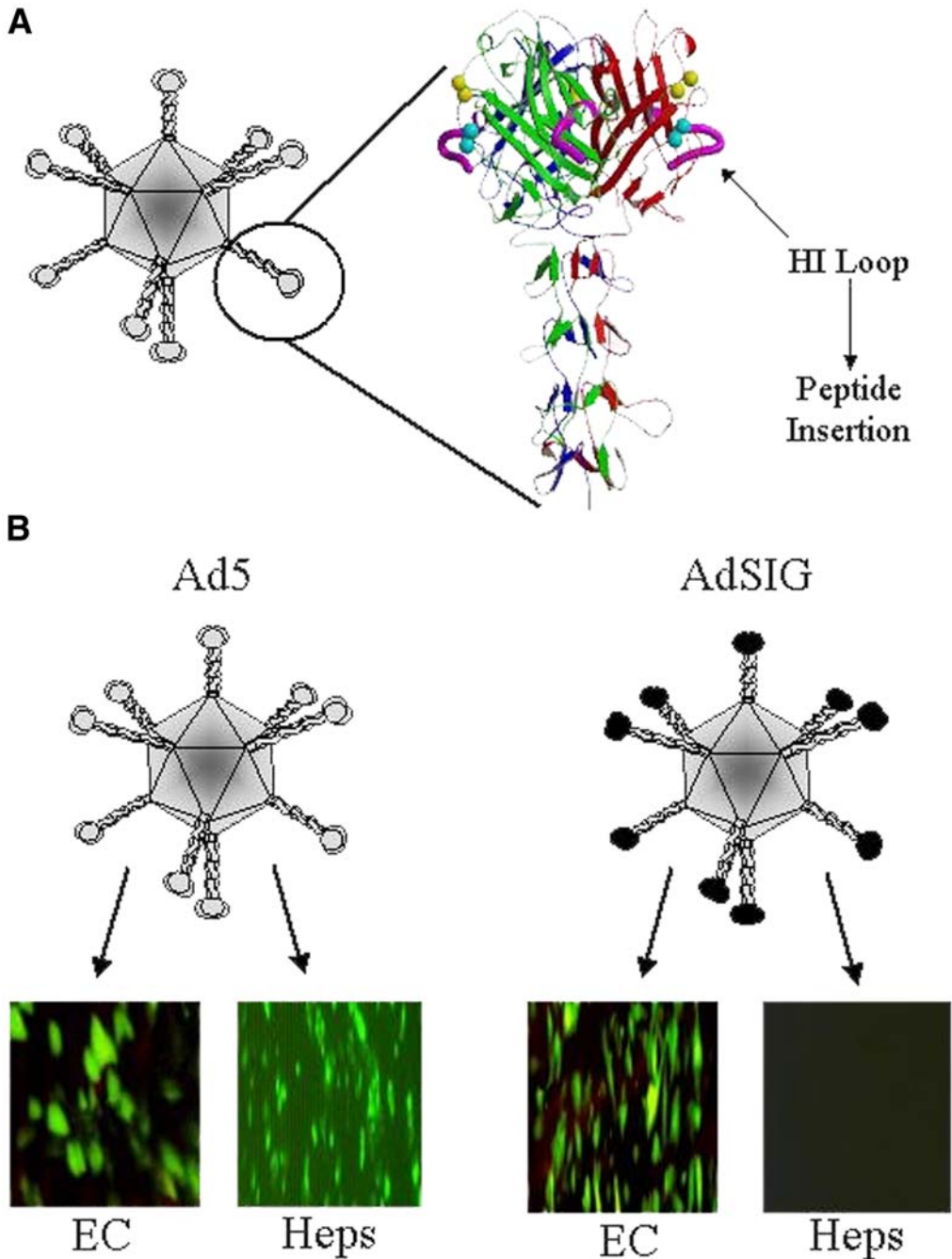
#### RETARGETING BY TROPISM ABLATION AND LIGAND INSERTION

The majority of studies aimed at incorporation of retargeting ligands now utilize a structural feature of the fiber protein, known as the HI loop (63), as a point of insertion. This loop is not important in maturation of the virus or in molecular interactions of the fiber with other domains (64), and has been demonstrated to be amenable to targeting peptide insertion in a number of studies. Many of these studies have utilized incorporation into the HI loop of the integrin-binding arginine–glycine–aspartate (RGD)–4C motif, originally isolated by *in vivo* phage display (65). This results in enhanced transduction of a number of cell and tissue types, including endothelial cells (66) and rabbit jugular vein grafts (67). Incorporation of the RGD motif into the hexon base of the Ad5 capsid produced a significantly increased level of transduction of human vascular smooth muscle cells (68). However, despite markedly more efficient gene transfer in vectors engineered to incorporate the RGD motif, a key limitation of this system is a lack of selectivity. This has led to the development of vectors engineered to include specific targeting sequences (typically identified using biopanning of random phage libraries) in the HI loop. This has been shown to be successful in a number of studies (69–72). In particular, it has been demonstrated that it is feasible to incorporate retargeting peptides, as well as mutations aimed at ablation of the natural tropism of the vector, resulting in selective and efficient transgene delivery into vascular endothelial cells (71; *see also* Fig. 3) and vascular smooth muscle cells (70) *in vitro*.

Intriguingly, the *in vivo* profile of modified vectors can be complex, often showing increased transgene expression in target tissues but unaltered natural tropism (i.e., high levels of hepatocyte transduction), in contrast with *in vitro* studies (72–74). Thus, it appears that yet further elucidation of the molecular basis for Ad5 binding and internalization is required in order to significantly ablate liver uptake and facilitate cardiovascular-specific targeting of this vector following systemic administration.

#### *Targeting of AAV*

Although fewer targeting strategies based on the AAV system are reported in the literature, parallel advances to those in Ad5 retargeting are being developed. Long-term expression and the ability to transduce nondividing cells makes the AAV-2 platform an attractive option for gene therapy in a number of particular pathologies. This is despite a significantly decreased ability, relative to adenovirus, to transduce endothelial cells, which are a key target in cardiovascular disease (36). The development of pseudotyping strategies or the adoption of alternative serotypes may eventually prove fruitful methods by which to retarget AAV-2. This observation is based in part on the range of different AAV serotypes, many of which exhibit variations in tropism (75). However, determination of the binding characteristics and infection pathways of these individual serotypes has yet to be described in detail, limiting the applicability of this approach. Some studies have begun to unravel this complexity of tropism in individual isoforms, demonstrating for example enhanced transduction of human endothelial and vascular smooth muscle



**Fig. 3.** Genetic targeting of Ad5 to EC. **(A)** Modification of the Ad fiber was achieved via insertion of the peptide SIGYPLP into the HI loop in combination with two amino acid substitutions in the fiber AB loop to ablate fiber: Coxsackie-adenovirus receptor interaction creating AdSIG(71). **(B)** Transduction of EC and hepatocytes (Heps) by AdSIG in comparison to Ad5. Viruses express enhanced green fluorescent protein as a reporter gene. Image of Ad5 fiber crystal structure courtesy of Vijay Reddy, The Scripps Research Institute, CA. Ad5, adenovirus serotype5.

cells in AAV-2 relative to AAV-3, -4, -5, and -6 (36). Further elucidation of the native tropism of these isoforms may eventually lead to the identification of serotypes specific for particular target cells.

Genetic modification of the AAV-2 capsid has also been described in the literature, and offers promise in the specific targeting of this vector. Since the identification of a site at position 587 on the AAV-2 capsid by Girod (76), which is optimal for the insertion of targeting peptides, this approach has been demonstrated to successfully enable targeting to a range of cell types, including human vascular endothelial (77) and smooth muscle (70) cells. The publication of the crystal structure of AAV-2 (78) has already led to the identification of a heparan-binding motif that contributes to cell binding (79). Subsequent studies seem likely to reveal further specific capsid epitopes and potential sites for modification of AAV-2 tropism.

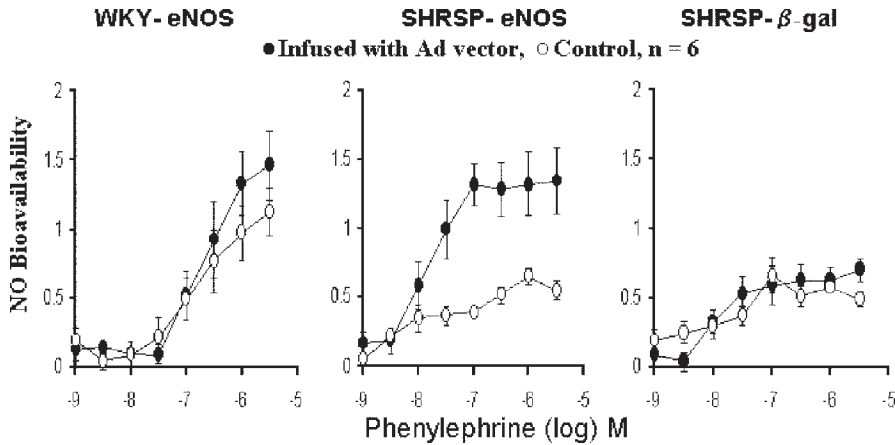
## APPLICATION OF GENE TRANSFER TO CARDIOVASCULAR DISEASE

A wide variety of cardiovascular pathologies are potential targets for gene therapy intervention. The majority of cardiovascular gene therapy studies are still based on pre-clinical models, although a number of clinical trials have begun to show promise. Some of the most frequently studied and clinically important cardiovascular diseases and gene therapy strategies aimed at overcoming them are outlined in this section.

### *Hypertension*

Primary systemic hypertension is a chronic elevation of blood pressure that is thought to be the result of interplay between polygenic and numerous environmental factors. Hypertension is a well established risk factor for secondary conditions, such as stroke, atherosclerosis, peripheral vascular disease, cardiac failure, and others (1). Although current pharmacological interventions are able to offer effective blood pressure control, such treatments are relatively short-acting, have a propensity for side effects, and rely heavily on good patient compliance for optimum benefit. In practice, these problems can mean that hypertension is often poorly controlled. Thus, gene therapy for the various features of hypertension has become increasingly explored as an alternative or adjunct to established pharmacological approaches.

The role of the renin-angiotensin system and its relationship with vascular oxidative stress have become well characterized features in the pathophysiology of hypertension. For example, it is now well established that reduced nitric oxide bioavailability and increased superoxide levels (via the actions of NADPH oxidase and uncoupled endothelial nitric oxide synthase [eNOS], among others) may underlie the vascular dysfunction that is a prominent feature of both human hypertension and animal models of the disease (80–82). Thus, various elements of this system are potential targets for gene transfer intervention and form the basis for the majority of gene therapy studies in relation to hypertension (83). However, probably resulting in part from the multifactorial nature of hypertension, clinical study of gene therapy for this condition has yet to be undertaken. Nonetheless, increasing experimental studies have shown much promise in this area. For example, it has been shown that local *in vivo* administration of adenoviral vectors encoding either eNOS (*see* Fig. 4) or extracellular superoxide dismutase (EC-SOD) can improve nitric oxide bioavailability and vascular function in the stroke-prone, spontaneously hypertensive rat (SHRSP) model (84,85). Similarly, eNOS overexpression was able to



**Fig. 4.** Improving vascular function in hypertensive rats using gene transfer. Adenoviral vectors encoding for endothelial nitric oxide synthase (eNOS) or the control reporter gene,  $\beta$ -galactosidase, were infused locally in vivo into the carotid artery of spontaneously hypertensive rat, stroke prone (SHRSP), or the normotensive reference strain, Wistar Kyoto (WKY). Basal nitric oxide (NO) bioavailability was determined as a function of the difference in contractile responses to phenylephrine in the presence and absence of the NOS inhibitor L-NAME. Overexpression of eNOS, but not  $\beta$ -galactosidase, in the carotid artery of SHRSP caused a significant augmentation in basal NO bioavailability, to levels comparable to the normotensive WKY strain. (Adapted from ref. 85.)

restore endothelial function in aortic rings in a model of angiotensin II-induced hypertension (86). Encouraging systemic hypotensive effects of gene transfer have also been demonstrated. For example, AdeNOS delivery into the rostral ventral medulla significantly lowered blood pressure in the spontaneously hypertensive rat (SHR) strain, as well as in the normotensive Wistar Kyoto strain (87). Intramuscular injection of adenoviral vector encoding tissue kallikrein inhibited the normal developmental increase in spontaneously hypertensive rat (SHR) blood pressure for a period of 5 wk (88), whereas infusion of naked human eNOS plasmid DNA reduced blood pressure for 6 wk in rats (89). More recently, systemic administration of AdEC-SOD was shown to significantly lower blood pressure in the SHR (90). Intriguingly, a heparin-binding domain-deleted version of this transgene—which should in theory produce a secreted product that is able to have more widely disseminated effects—did not affect blood pressure in this latter study, showing that further understanding of the molecular activity of putative therapeutic agents may be required. Recent work from our own group has shown that systemic administration of a retargeted adenovirus expressing eNOS (based on the previous work of Reynolds and others [56–58]) can lower systolic blood pressure in the SHR, stroke prone model (see Fig. 2). A more novel approach has been the use of AAV-mediated delivery of antisense angiotensin 1 receptor into cardiac tissue of SHR, producing a reduction in blood pressure of 30 mmHg (91). As the fine details of the molecular pathways involved in maintaining vascular function become increasingly well defined, so the number of potential gene therapy targets increases (92). For example, it has recently been demonstrated that gene transfer of human guanosine 5'-triphosphate cyclohydrolase I, which is the initial and rate-limiting step in the generation of the essential eNOS cofactor tetrahydrobiopterin, can correct vascular superoxide levels in a rat model of hypertension (93). Furthermore, the identification of putative candidate genes involved in the devel-

opment of hypertension using congenic breeding strategies and gene microarray techniques (94) has been the subject of recent studies, and is now beginning to prove fruitful (95). Further characterization of such candidates will present further targets for hypertension gene therapy.

### ***Late Vein Graft Failure***

Coronary artery bypass grafting (CABG) is a procedure that successfully alleviates angina pectoris and increases life expectancy in patients with coronary artery disease. However, the vein graft undergoes adaptive responses to transplantation into the arterial circulation, with early thrombus formation occurring in 10% of grafts, and patency dropping to 50% at 10 yr postprocedure. Vein graft failure is associated with progressive intimal thickening, atheroma development, and neointima formation (96), with associated effects on morbidity and mortality after CABG. Drug treatments (typically antiplatelet agents) can slow and reduce this process, but can be associated with hemorrhagic events. However, vein grafting is particularly amenable to gene transfer, as the vein graft itself is isolated and able to undergo genetic modification during the surgical procedure. This concept was demonstrated clearly in a clinical trial in which vein grafts transduced during the procedure with vectors expressing decoy oligodeoxynucleotides against the transcription factor E2F showed significantly reduced postoperative occlusion (6). While research into E2F decoy administration continues, the majority of transgenes investigated in other studies have been chosen for their antiproliferative, antiinflammatory, proapoptotic, or antimigratory agents (97).

A key component in the remodeling of the failing vessel is the migration and proliferation of smooth muscle cells, a feature partly determined by the activity of the matrix metalloproteinases. Adenovirally mediated overexpression of the endogenous tissue inhibitors of MMPs has shown beneficial effects, such as reduction of neointima and inhibition of lesion development in a number of studies of vein graft failure, including porcine *in vivo* and human *ex vivo* models (7–9). Other transgenes that have shown promise include nitric oxide synthase (98), p53 (99), retinoblastoma gene product (100) and natriuretic peptide (101). The majority of these studies have examined endpoints measured in days or weeks, although significantly reduced neointima and increased vessel patency at 3 mo was recently demonstrated in a porcine model following adenovirally mediated p53 overexpression (102). However, the long-term nature of vein graft failure requires gene therapy capable of producing long-lasting effects, a feature that remains to be fully demonstrated in this setting.

### ***Hypercholesterolemia and Atherosclerosis***

Familial hypercholesterolemia is caused by the absence of or a defect in the LDL receptor. This results in an inability to clear LDL from the bloodstream, leading to severe hypercholesterolemia and accelerated atherosclerosis. Various models of this condition have been studied. Gene transfer of the corrected LDL receptor has been shown to be beneficial in both murine and human studies (5,103–106). Furthermore, it has been shown that delivery of the very low-density lipoprotein (VLDL) receptor (using modified helper-dependent adenoviral vectors) into the LDL receptor-deficient mouse model (107) could produce sustained lipid-lowering effects for up to 6 mo. Even more promising, in an *ApoE*-deficient mouse model, delivery of the *ApoE* gene using a helper-dependent adenoviral vector (18) resulted in decreased serum lipid concentrations and virtually

complete absence of aortic lesions for the lifetime of the animals (approx 2.5 yr). This was in contrast with complete coverage of control aortae by atherosclerotic plaque. Although such studies are very encouraging (and indeed also highlight the choice of appropriate vector), it remains to be determined how these findings may relate to the clinical situation, where atherosclerotic plaque development may already be well advanced before treatment is commenced.

The multifactorial nature of the development of atherosclerosis, and in particular the inextricable interaction between environmental and genetic factors, make utilization of gene therapy approaches a difficult challenge. This may underlie, in part, the relatively few successful studies of gene therapy approaches as an intervention for established atherosclerosis, with many studies instead focusing on primary conditions, such as hypercholesterolemia. It has been suggested that low gene-transfer efficiency across atherosclerotic plaques and atheroma may be as a result of the high level of connective tissue and the low number of transducible cells (108). Nonetheless, there are reports of potentially beneficial results utilizing gene transfer for expression of a number of proteins involved in lipid metabolism, including apolipoprotein AI, lecithin-cholesterol acyltransferase (109), and heme oxygenase (110).

### *Ischemic Disease and Reperfusion Injury*

Ischemic disease can affect both the peripheral and coronary circulations, and is manifest as reduced blood flow resulting from the presence of thrombus, atherosclerotic plaque, or vascular spasm. This has the effect of starving tissues distal to the narrowing of oxygen and other nutrients. Although surgical bypass and angioplasty are able to provide symptomatic relief, this tends to be temporary in nature and is not suitable for all patients. Thus, the only long-term solution in some cases may ultimately be the amputation of affected limbs or heart transplantation. Gene therapy may therefore offer a promising alternative for treatment of these conditions (111,112). Most studies in this area have focused on delivery of VEGF and fibroblast growth factor (FGF), two angiogenic growth factors, to the ischemic areas. Furthermore, because VEGF is a secreted protein, its physiological effect may extend beyond transduced cells, reducing the requirement to transduce as many cells as possible.

Intramuscular injection of adenoviral (113) and AAV (31) vectors expressing VEGF has been shown to induce angiogenesis, improve peripheral blood flow, and also be protective in animal models of limb ischemia. Furthermore, adenovirally mediated delivery of VEGF was shown to improve endothelial function and lower extremity blood flow in humans with peripheral artery disease (114). Similar effects have been observed in ischemic heart disease in humans—for example, alleviation of angina and angiographic improvement of vessel narrowing—following direct myocardial injection of VEGF vectors (115). Clinical trials of adenoviral delivery of FGF-4 improved patient exercise tolerance, with an absence of adverse effects (116) and increased myocardial perfusion, while reducing ischemic defect size (117).

Although these preclinical and early clinical studies of gene therapy for ischemia show promise, concerns still exist over the safety of overexpression of angiogenic factors, such as VEGF. For example, it has been suggested that VEGF may enhance the development of atherosclerosis (118), stimulate the generation of dysfunctional vessels (119), and promote formation of vascular tumours (120). More recently, it has been shown that the microenvironment concentration of VEGF, rather than the overall dose, is of extreme

importance in determining whether its effects are normal or aberrant (121). Specific targeting of vectors and tight regulation of transgene expression, therefore, seem necessary to further improve the safety profile of these vectors. Following an ischemic episode, reperfusion of the myocardium can itself lead to further injury of the ischemic zone, in a process that has been shown to involve oxidative stress. Gene therapy strategies utilizing expression of antioxidants and other agents, including superoxide dismutase (122) and heme oxygenase (123), have shown promise in animal models. Refinement of such approaches to include a myocardium-specific promoter and a hypoxia-regulatory element (to switch on transgene expression only under hypoxic conditions), has also been described (124).

## CONCLUSIONS

The past decade has seen an explosion in gene therapy studies, and it is now clear that this field offers promise in terms of new approaches to molecular medicine. Application of gene transfer as an approach to cardiovascular disease is a complex matter, because of the multifactorial nature of such diseases. It is therefore clear that further elucidation of the pathophysiology of cardiovascular diseases, and thus identification of key molecular genetic targets, coupled with the further development of safe, efficient, targeted gene transfer vectors, are key requirements. Further development of methods of gene suppression, such as antisense technology and RNA interference, and their incorporation into gene therapy vectors will offer an alternative to overexpression as a means of intervention. Progress in these areas will ultimately determine if the promise shown in both laboratory studies of gene therapy and in initial clinical trials is to translate into a standard therapeutic approach for cardiovascular disease.

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## Kallikrein Gene Transfer in Hypertension, Cardiovascular and Renal Disease, and Stroke

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*Julie Chao, PhD and Lee Chao, PhD*

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## SUMMARY

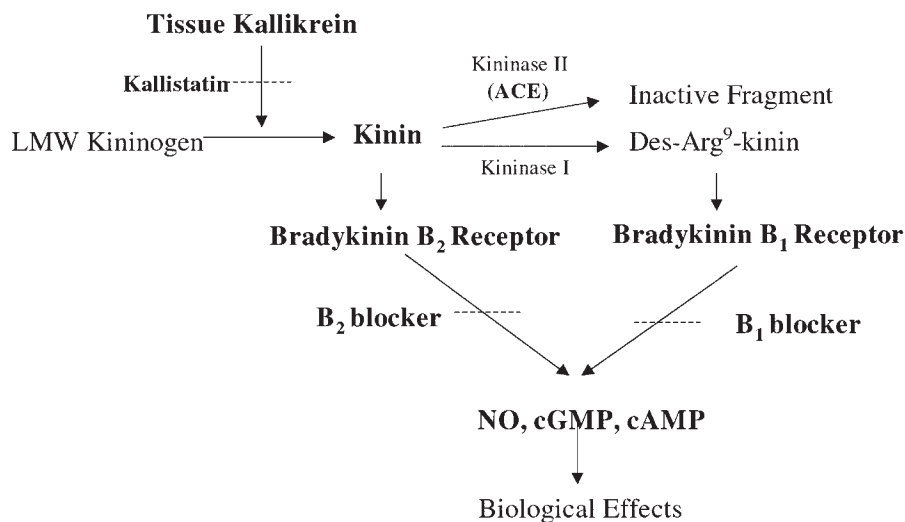
Tissue kallikrein cleaves low-molecular-weight kininogen substrate to produce the potent vasodilator kinin peptide, which binds to kinin receptors and triggers a wide spectrum of biological effects. Tissue kallikrein levels are reduced in humans and animal models with hypertension and cardiovascular and renal disease. We investigated the role of the tissue kallikrein–kinin system in the cardiovascular, renal, and central nervous systems by systemic and local delivery of the human tissue kallikrein gene using a plasmid DNA or an adenovirus vector. Enhanced kallikrein/kinin levels following kallikrein gene transfer reduce blood pressure in several pressure- and volume-overload hypertensive animal models. Kallikrein gene transfer also exerts beneficial effects in protection against cardiac remodeling, renal fibrosis, restenosis, cerebral infarction, and neurological deficits. The improvement in cardiovascular, renal, and neurological function following kallikrein gene transfer is independent of kallikrein's ability to lower blood pressure. Kallikrein gene transfer has pleiotropic effects on apoptosis, inflammation, proliferation, hypertrophy, fibrosis, and angiogenesis in the heart, blood vessel, kidney, and brain. These effects are blocked by icatibant, a bradykinin B2 receptor antagonist, indicating a kinin-mediated event. Mechanistically, kallikrein gene transfer leads to increased nitric oxide (NO) levels and decreased oxidative stress and inflammatory response. These novel findings indicate that kallikrein/kinin, through NO formation, may act as an antioxidant and anti-inflammatory agent in protection against cardiovascular and renal disease in animal models, and may uncover new drug targets for the prevention and treatment of heart failure, end-stage renal disease, and stroke in humans.

**Key Words:** Tissue kallikrein; kinin; gene therapy; hypertension; oxidative stress; heart; kidney; blood vessel; brain.

## THE TISSUE KALLIKREIN–KININ SYSTEM COMPONENTS

Tissue kallikreins (E.C. 3.4.21.35) belong to a subgroup of serine proteinases that process kininogen substrates and release vasoactive kinin peptides (1). The well-recognized function of tissue kallikrein is mediated by lysyl-bradykinin (Lys-BK or kallidin) and bradykinin (BK), which consist of 10 and 9 amino acid peptides, respectively. Kinins are then degraded by enzymes, such as kininases I and II and neutral endopeptidase to produce a number of kinin metabolites or inactive fragments. Intact kinins bind to BK B2 receptors, whereas kinin metabolites, such as Des-Arg<sup>9</sup>-BK or Des-Arg<sup>10</sup>-Lys-BK, bind to BK B1 receptors. The physiological functions of the tissue kallikrein–kinin system (KKS) are mediated by the constitutively expressed B2 receptor. Unlike the B2 receptor, the B1 receptor is expressed at low levels in the heart, vasculature, and kidney and is induced by trauma or inflammation (2). The binding of kinins to their respective receptors activates second messengers, such as nitric oxide (NO), cyclic adenosine monophosphate (cAMP), prostacyclin, and cyclic guanosine monophosphate (cGMP), which trigger a broad spectrum of biological effects including vasodilation, smooth muscle contraction and relaxation, inflammation, and pain (3,4).

Regulation of the KKS exists through system-specific inhibitors, kininases, and antagonists, as well as through a kininase shared with the renin–angiotensin system (RAS) (Fig. 1). The KKS can be blocked by tissue kallikrein inhibitors (kallistatin or aprotinin), icatibant (Hoe 140, a specific B2 receptor antagonist), or Des-Arg<sup>9</sup>-[Leu<sup>8</sup>] BK (a specific B1 receptor antagonist) (1,3). Kallistatin is a serine proteinase inhibitor (serpin) that forms a covalently linked complex with tissue kallikrein, resulting in inhibition of tissue kallikrein activity (5–7). The KKS and RAS are linked by angiotensin-converting enzyme (ACE), a dipeptidase, which is the same enzyme as kininase II. ACE has dual



**Fig. 1.** The tissue kallikrein–kinin system. ACE, angiotensin-converting enzyme; LMW, low-molecular-weight; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; cAMP, cyclic adenosine monophosphate.

functions: it not only converts angiotensin I to vasoconstrictor peptide angiotensin II (Ang II), but also degrades kinin to release a dipeptide fragment, Phe-Arg, from the carboxyl end of the kinin peptide, rendering kinin inactive. Therefore, the beneficial effects of ACE inhibition in hypertension and cardiovascular and renal disease can also be attributed to kinin accumulation, as icatibant can partially abolish these effects (8–10). Thus, the vasodilator action of the KKS can counter-regulate the vasoconstrictive action of the RAS.

## TISSUE KALLIKREIN IN HYPERTENSION

Hypertension is a major disease that affects approx 25% of the population in the United States. African Americans are more likely to develop salt-sensitive hypertension. A previous study suggested that 50–75% of the hypertensive population demonstrates salt sensitivity, as blood pressure can be altered in response to changes in dietary salt intake (11). Moreover, hypertensive individuals are more likely to develop other cardiovascular and renal disorders, such as peripheral vascular disease, coronary heart disease, congestive heart failure, and renal disease. Hypertension could result from either an excess of vasoconstrictor substances or a deficiency in vasodilator substances. Abnormality of renal kallikrein levels has long been documented in the pathogenesis of hypertension (12,13). Epidemiological studies have identified an inverse relationship between urinary or renal kallikrein levels and blood pressure in patients with essential hypertension (14–16). Because urinary kallikrein originates in the kidney, reduced urinary kallikrein levels would suggest impaired renal function. An association between reduced excretion of kallikrein and human hypertension has been reported both in Caucasian and African-American individuals (13–15). It has been shown that the influence of both race and dietary sodium intake upon kallikrein excretion in hypertensive patients is greater than that of normotensive individuals (13–15). Furthermore, a study aimed at identifying genetic factors associated with cardiovascular risks using highly informative family pedigrees indicated that



a dominant gene expressed as renal or urinary kallikrein may be associated with a reduced risk of hypertension (16). This finding suggests that high urinary kallikrein may have a protective effect against the development of high blood pressure.

Reduced urinary kallikrein excretion has also been described in a number of genetically hypertensive rat models such as spontaneously hypertensive rats (SHR), New Zealand rats, Fawn-Hooded rats, and Dahl salt-sensitive (DSS) hypertensive rats (17–21). Kinin appears to play a role in blood pressure regulation in SHR fed a salt-deficient diet, because the administration of a BK antagonist caused an increase in blood pressure (22). Furthermore, kininogen-deficient (Brown Norway–Katholiek) rats, which cannot generate kinin, are susceptible to the development of salt-induced hypertension (23). Several restriction fragment length polymorphisms have been mapped in the rat tissue kallikrein gene, and their regulatory regions have been identified in the SHR model (24). These findings indicate a possible difference in the tissue kallikrein gene locus between SHR and normotensive Wistar–Kyoto rats. Moreover, a tissue kallikrein RFLP has been shown to cosegregate with high blood pressure in the F2 offspring of SHR and normotensive Brown Norway–Katholiek crosses (25), suggesting a close linkage between the kallikrein gene locus and the hypertensive phenotype in SHR.

We have shown that transgenic mice and rats overexpressing human tissue kallikrein were permanently hypotensive throughout their lifetime compared with their control littermates (26–29). Administration of aprotinin or icatibant to the transgenic mice raised their blood pressures to normal levels (26,27). These results indicate that the expression of functional human tissue kallikrein can permanently alter the blood pressure setting in the transgenic animals, and that the effect is mediated by the BK B2 receptor. This conclusion is further reinforced by a study showing that transgenic mice overexpressing human BK B2 receptor are hypotensive when compared with their control littermates (30). Moreover, ablation of the B2 receptor gene in mice causes salt-sensitive hypertension (31). Taken together, these findings provide the first direct evidence demonstrating the role of the KKS in blood pressure regulation *in vivo* and lend support to the hypothesis that elevated levels of tissue kallikrein may offer a protective effect in preventing the development of hypertension and cardiovascular and renal complications.

### SOMATIC GENE DELIVERY OF TISSUE KALLIKREIN IN HYPERTENSIVE ANIMAL MODELS

One strategy to study the potential role of kallikrein/kinin in hypertension is via protein or gene delivery. Oral administration of purified pig pancreatic kallikrein has been used to temporarily lower the blood pressure of hypertensive patients (32,33). This hypotensive effect requires repeated administrations of the enzyme, and the antihypertensive effect disappears shortly after the treatment is discontinued (32,33). Somatic gene delivery results in continuous expression of the gene of interest for an extended period of time, and is a potential therapeutic tool in treating cardiovascular and renal disease. To investigate the impact of supplying tissue kallikrein by gene delivery, we generated a series of human tissue kallikrein gene constructs under the control of the metallothionein metal response element, the albumin promoter, the Rous sarcoma virus 3' long terminal repeat, or the cytomegalovirus (CMV) promoter (28). Injection of these plasmid cDNA constructs into SHR caused a prolonged reduction of systemic blood pressure for several weeks (34–36). The extent of blood pressure reduction was dependent on the dose of DNA injected, the gender of the animals, the promoter used in the gene construct, and the route of injection (34–36).

To achieve high-efficiency expression of tissue kallikrein in animal models, a transgene expression cassette containing the human tissue kallikrein cDNA under the control of the CMV promoter and 4F2 enhancer was inserted into an adenovirus vector and then transfected into human embryonic kidney 293 cells. Expression of human tissue kallikrein was detected in the culture media by a specific enzyme-linked immunosorbent assay (ELISA). We have shown that a single intravenous injection of adenovirus encoding the human tissue kallikrein gene resulted in a rapid and profound reduction of blood pressure for weeks in SHR, DSS, deoxycorticosterone-acetate (DOCA)-salt, 2 kidney 1 clipped (2K1C), and 5/6 nephrectomy rats (37–41), compared with rats injected with control adenovirus. Kallikrein gene transfer was also effective in causing a prolonged reduction of blood pressure using several nonsystemic delivery routes, including intramuscular, intraportal vein, and intracerebroventricular injections (34,36,42). The expression of recombinant human tissue kallikrein in rats following systemic or local gene delivery was detected by specific ELISA and immunohistochemistry, as well as by reverse transcription-polymerase chain reaction followed by Southern blot analysis. Immunoreactive human tissue kallikrein levels in rat sera or urine were highest at 3–5 d after adenovirus-mediated gene delivery, and were still detectable at 24 d after injection (37). However, no human tissue kallikrein was detected in the urine or sera of rats injected with control virus. The kallikrein inhibitor aprotinin reversed the blood pressure-lowering effect in SHR receiving kallikrein gene transfer (35). These results indicate that the hypotensive effect of kallikrein gene delivery is mediated by the expression of functional kallikrein. The effect of kallikrein on blood pressure reduction can also be reversed by icatibant, suggesting that the effect is dependent on a BK B2 receptor-signaling event (29).

## KALLIKREIN/KININ AND CARDIAC PROTECTION

The KKS has been demonstrated to play a vital part in cardiac protection in a variety of animal models. Studies using ACE inhibitors clearly showed a protective effect of endogenous kinins in the development of cardiac hypertrophy and neointimal vascular injury (43,44). By employing transgenic and somatic gene transfer approaches, we demonstrated that the KKS plays an important role in cardiac protection. Overexpression of human tissue kallikrein in transgenic rats resulted in reduction of isoproterenol-induced cardiac hypertrophy and fibrosis, and these effects were abolished by icatibant (29). In addition, ablation of the B2 receptor gene in mice caused dilated cardiomyopathy followed by cardiac failure (45). Moreover, systemic delivery of adenovirus containing the tissue kallikrein gene led to blood pressure reduction and attenuation of cardiac hypertrophy and fibrosis in pressure- and volume-overload hypertensive rat models, such as SHR, 2K1C, DSS, and DOCA-salt rats (37–40). Our recent studies also showed that kallikrein gene transfer attenuated cardiac hypertrophy and fibrosis in normotensive rats after myocardial infarction and in genetically hypertensive rats without apparently affecting blood pressure (46,47). These results indicate that kallikrein/kinin not only plays an important role in the regulation of blood pressure homeostasis, but also in cardiovascular function.

## KALLIKREIN/KININ ATTENUATES CARDIAC REMODELING AFTER MYOCARDIAL INFARCTION

ACE inhibition has been shown to improve the survival of rats with coronary ligation-induced myocardial infarction and with congestive heart failure (9,10). ACE inhibitors

have many beneficial effects in respect to the reduction of morbidity and mortality rates and improvement of the quality of life for patients with heart disease (48,49). Ang II formation in the heart increases after myocardial infarction (50), and elevation of Ang II levels causes cardiac hypertrophy, fibrosis, and apoptosis, leading to cardiac remodeling and heart failure (50–53). A continuous supply of kallikrein/kinin may counteract the action of Ang II and thus protect against cardiac remodeling and heart failure. Our studies showed that kallikrein gene transfer improved cardiac and endothelial function and attenuated myocardial infarction in normotensive animals with acute ischemic and chronic heart disease (46,54). Kallikrein gene delivery reduced cardiac remodeling and apoptosis after myocardial infarction and delayed the progression of heart failure without changes in blood pressure (46,54). The cardioprotective effects mediated by kallikrein gene transfer include: (1) improved cardiac function and cardiac reserve in response to stress; (2) reduced cardiac hypertrophy, fibrosis, and left-ventricular enlargement; (3) improved endothelial function by increasing blood flow and decreasing vascular resistance; (4) increased capillary density in the heart; (5) reduced oxygen consumption; and (6) reduced myocardial apoptosis. This study indicates that the KKS plays an important role in preventing the progression of heart failure after myocardial infarction in chronic heart disease.

### INHIBITION OF CARDIOMYOCYTE APOPTOSIS IN ISCHEMIC MYOCARDIUM BY KALLIKREIN GENE DELIVERY

In rats subjected to acute ischemia/reperfusion (I/R), systemic delivery of the tissue kallikrein gene exerts cardiac protection by reducing myocardial infarction, the incidence of ventricular fibrillation, and apoptosis (54,55). Icatibant abolished these beneficial effects, indicating a kinin-mediated event. To achieve a high level of heart-specific expression of recombinant proteins, we employed a catheter-based gene delivery technique to deliver the kallikrein or reporter gene locally into the left ventricle (55). The expression and localization of immunoreactive human tissue kallikrein was identified throughout the left ventricle after local delivery of the kallikrein gene. No specific staining was found in rats injected with control adenovirus (55). In addition, local delivery of adenovirus harboring the green fluorescent protein gene showed homogenous distribution of green fluorescence in the left ventricle, whereas the control left ventricle showed little background fluorescence (56). Moreover, after injection of the adenovirus encoding the luciferase gene, the highest level of luciferase activity was found in the left ventricle with very low levels in the right ventricle, aorta, and other tissues, indicating left ventricle-specific expression (56). Similar to systemic gene delivery, local kallikrein gene delivery significantly reduced I/R-induced myocardial apoptosis in the left ventricle, as detected by both the terminal deoxynucleotidyl transferase-mediated nick end-labeling (TUNEL) staining and DNA laddering.

Akt is a serine/threonine kinase that has been shown to be a key player in promoting cell survival and suppressing apoptosis. Akt promotes survival of cardiomyocytes *in vitro*, and protects against I/R-induced injury in the mouse heart (57,58). Akt inhibits apoptosis by phosphorylating Bad, leading to increased Bcl-2 and Bcl-xL levels and cell survival (59,60). We showed that kallikrein gene transfer inhibited I/R-induced cardiomyocyte apoptosis, which was accompanied by activation of Akt, Bad, and increased Bcl-2 levels, indicating a role for Akt-Bad-Bcl-2 signaling in kallikrein-mediated protection against myocardial apoptosis (55). Moreover, glycogen synthase kinase-

3 $\beta$  (GSK-3 $\beta$ ) is also a downstream target of Akt signaling. Phosphorylation of GSK-3 $\beta$  by Akt inhibits its activity in insulin-mediated signal transduction (61). Kallikrein gene transfer led to increased phosphorylation of GSK-3 $\beta$  and reduced GSK-3 $\beta$  activity, as well as decreased caspase-3 activation in the ischemic myocardium and in primary cultured cardiomyocytes subjected to hypoxia/reoxygenation. Kallikrein's protective effect on apoptosis was blocked by icatibant and by a dominant-negative Akt construct (55). These results indicate that kallikrein/kinin protects against cardiomyocyte apoptosis via activation of Akt-Bad and Akt-GSK-3 $\beta$  signaling pathways.

### PROTECTION OF VASCULAR INJURY BY KALLIKREIN GENE DELIVERY

We have shown that transfection of the kallikrein gene or kinin peptide inhibited the proliferation of cultured vascular smooth muscle cells (62). Transfection of the kallikrein gene into isolated aortic segments resulted in a time-dependent secretion of recombinant human tissue kallikrein, which coincided with significant increases in NO and cGMP levels (63). To further investigate the role of kallikrein/kinin in vascular injury, human tissue kallikrein gene was delivered locally into the rat's left common carotid artery after balloon angioplasty. Kallikrein gene delivery resulted in a significant reduction in intima/media ratio at the injured vessel compared with the rats receiving control virus (62,63). The inhibitory effect of kallikrein on neointima formation was blocked L-NAME, a NO synthase inhibitor, and by icatibant, indicating a kinin-B2-receptor-NO-dependent event (62,63). Moreover, systemic delivery of the kallikrein gene into a mouse model of arterial remodeling induced by permanent alteration in shear-stress conditions resulted in a reduction of neointima formation (64). The protective action of kallikrein gene transfer was significantly reduced in kinin B2 knockout mice, but amplified in transgenic mice overexpressing human kinin B2 receptor, compared with wild-type mice (64). In streptozotocin-induced diabetic mice, local delivery of the human tissue kallikrein gene halted the progression of microvascular rarefaction in hindlimb skeletal muscle by inhibiting apoptosis and promoting vascular regeneration (65). Taken together, these results provide new insights into the role of the KKS in the vasculature and may have significant implications for a therapeutic application to treat restenosis and atherosclerosis.

### RENAL PROTECTION BY TISSUE KALLIKREIN AND KININ

The link between renal function and the KKS has been demonstrated by several studies. Renal kallikrein excretion has been reported to be significantly reduced in patients with mild renal disease, and more markedly reduced in patients with severe renal failure (66,67). Genetic linkage analysis has shown that a polymorphic site in the proximal promoter region of the human tissue kallikrein gene is associated with the development of end-stage renal disease and hypertension (68,69). Additionally, the kallikrein promoter polymorphism participates in regulating gene expression and modifies blood pressure in response to dietary salt intake (70). These findings suggest that expression of the kallikrein gene may serve as a powerful marker for linkage analysis in populations with salt-sensitive hypertension and renal disease. A variety of studies, including our own, indicate a direct protective effect of the KKS in the kidney. A previous report by other investigators showed that intravenous infusion of kallikrein protein via an osmotic minipump reduces renal damage without affecting the blood pressure of DSS rats fed a

high-salt diet (71). Renal protection by kallikrein infusion was abolished by icatibant, indicating a role of the KKS in protection against salt-induced renal injury through mechanisms independent of blood pressure reduction. Using a somatic gene transfer approach, we demonstrated that expression of human tissue kallikrein resulted in enhanced renal function by increasing the glomerular filtration rate and renal blood flow, and reducing glomerular sclerosis, tubular dilatation, and luminal protein cast accumulation (72). These beneficial effects were in conjunction with a partial reduction of blood pressure. Moreover, kallikrein gene transfer also reduced gentamicin-induced nephropathy in a normotensive animal model without affecting hemodynamic parameters (73). These results indicate that the KKS could protect against renal injury through mechanisms independent of its ability to lower blood pressure.

### TISSUE KALLIKREIN PROTECTS AGAINST SALT-INDUCED RENAL INJURY

Progressive renal injury is the consequence of a process of destructive fibrosis. High salt intake induces hypertension, cardiac hypertrophy, and progressive renal damage. DSS rats represent a model of progressive and sclerotic renal lesions and have been regarded as the closest model of human salt-sensitive hypertension. After high salt intake, DSS rats develop hypertension, severe renal damage, cerebral infarction, and hemorrhage (38,72,74). An increase in apoptosis in the glomerular and tubular compartments has also been observed in DSS rats (75). This event occurred at a time when renal function was markedly impaired and irreversible changes in renal morphology had developed, indicating a pathological role for apoptosis in renal dysfunction and fibrosis. We have shown that kallikrein gene transfer reduced salt-induced hypertension, glomerular sclerosis, and tubulointerstitial damage in DSS rats (38,72). In addition, we recently demonstrated that kallikrein gene delivery in DSS rats attenuated glomerular and tubular fibrosis scores, interstitial collagen accumulation, renal cell proliferation, glomerular hypertrophy, and apoptosis induced by high salt intake. These effects correlated with increased NO bioavailability and suppression of oxidative stress and transforming growth factor (TGF)- $\beta$ 1 expression. Based on these observations, kallikrein gene transfer appears to exert pleiotropic effects on the kidney of DSS rats.

### KALLIKREIN/KININ PROTECTS AGAINST GENTAMICIN-INDUCED NEPHROTOXICITY

Gentamicin is an aminoglycoside antibiotic commonly used in treating life-threatening Gram-negative bacterial infections. However, gentamicin treatment causes serious complications, such as nephrotoxicity and ototoxicity. Gentamicin treatment has been reported to cause apoptosis in both cultured renal proximal tubules and auditory hair cells (76,77), as well as inflammation in the kidney (78). We have demonstrated that kallikrein gene transfer enhances renal function and reduces blood urea nitrogen in gentamicin-induced nephrotoxic rats (73). Moreover, kallikrein gene transfer significantly attenuated gentamicin-induced glomerular sclerosis and tubular dilatation, inflammatory cell (macrophage/monocyte) infiltration, and TUNEL-positive apoptotic cells in both the cortex and medulla. These protective effects were accompanied by increased NO formation and reduced nicotinamide adenine dinucleotide oxidase activity, superoxide formation, intercellular adhesion molecule-1, and TGF- $\beta$  levels, as well

as c-jun N-terminal kinase phosphorylation. Kallikrein's effects on these signaling events were blocked by icatibant, indicating a kinin-mediated event. This study indicates that kallikrein/kinin protects against gentamicin-induced nephrosclerosis by inhibiting inflammatory cell recruitment, and against apoptosis through suppression of oxidative stress-mediated signaling pathways.

### KALLIKREIN GENE DELIVERY PROTECTS AGAINST PRESSURE-BASED STROKE

Hypertension is critical in the development of stroke in humans. In stroke-prone, spontaneously hypertensive rats (SHRSP), high salt intake accelerates the development of malignant hypertension (79). In the brains of SHRSP, fibrinoid necrosis and associated thrombosis primarily affect cerebral arterioles, leading to their obstruction and infarction, whereas cerebral hemorrhage is caused by microaneurysms (80). In addition, a lethal form of hypertension has been shown to develop in DSS rats fed a high-salt diet at an early age (81). Pathological changes in the brain of DSS rats affected by stroke include hemorrhage, edema, and infarction. We showed that a single injection of adenovirus carrying the human tissue kallikrein gene significantly reduced hypertension, stroke-induced mortality, aortic hypertrophy, and cerebral hemorrhage in DSS rats on a high-salt diet (74). These findings indicate that kallikrein/kinin may play a protective role in pressure-based stroke.

### KALLIKREIN GENE DELIVERY PROTECTS AGAINST ISCHEMIC STROKE

Stroke is the third leading cause of death, and the most common cause of long-term disability, in the United States (82). Currently, therapeutic options for the treatment of ischemic stroke are limited primarily to agents that block platelet aggregation or the coagulation cascade (83). However, these antiplatelet agents are only effective in decreasing the incidence of ischemic stroke, and not in reducing cerebral infarct size (83). Stroke-induced neurological deficits and mortality are often associated with timing of treatment after the onset of stroke. The therapy approved for the treatment of acute ischemic stroke is intravenous recombinant tissue-type plasminogen activator initiated within 3 h of symptom onset (84). Focal brain ischemia is the most common event leading to stroke in humans. To prevent or reduce irreversible ischemic brain damage, it is necessary to develop new interventions for acute stroke therapies to meet the large need for this important and undertreated disorder.

To determine the potential protective role of kallikrein/kinin in ischemic stroke, we employed a focal cerebral ischemic rat model with middle cerebral artery occlusion. Intracerebroventricular injection of adenovirus containing the human tissue kallikrein gene significantly reduced ischemia-induced locomotor deficits and cerebral infarction after focal cerebral ischemia. Expression of recombinant human tissue kallikrein in rat ischemic brain was identified by both immunohistochemistry and ELISA. Kallikrein gene transfer improved neurological function and enhanced the survival and migration of glial cells into the ischemic penumbra and core (85). Neuroprotection after kallikrein gene transfer was accompanied by markedly increased cerebral NO levels, phosphorylated Akt and Bcl-2 levels, and reduced nicotinamide adenine dinucleotide phosphate oxidase activity and caspase-3 activation (85). These results indicate that local delivery

of the kallikrein gene provides neuroprotection against focal ischemic brain injury by enhancing glial cell survival and inhibiting neuronal apoptosis through suppression of oxidative stress and activation of the Akt-Bcl-2 signaling pathway.

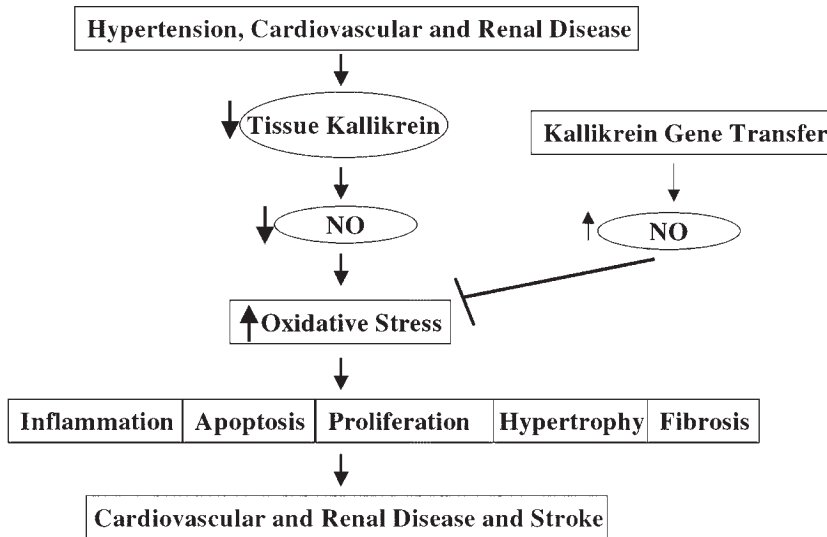
We also employed a more practical approach to further investigate the potential therapeutic time window for ischemic stroke by intravenous injection of the human tissue kallikrein gene. It has been reported that cerebral ischemia disrupts the blood–brain barrier and allows increased vascular permeability for up to 46 h (86). By taking advantage of this increased permeability, adenovirus containing the kallikrein gene was delivered intravenously to determine whether the kallikrein gene could reach damaged brain tissues to exert protection against I/R-induced cerebral injury. We showed that systemic delivery of the kallikrein gene before or after cerebral ischemia was effective in reducing locomotor deficit scores and cerebral infarction. Similar to local gene delivery, systemic delivery of the kallikrein gene attenuated neuronal apoptosis and promoted glial cell survival in the ischemic penumbra and core. In addition, kallikrein gene transfer promoted angiogenesis and neurogenesis in the brain after cerebral I/R. Icatibant blocked the kallikrein-mediated effects. These results indicate that systemic delivery of the tissue kallikrein gene after the onset of acute ischemic stroke can still provide protection against ischemia-induced brain injury. Taken together, our findings provide new and significant insights into potentially extending the time window for the treatment of ischemic stroke by kallikrein/kinin using a convenient delivery method.

### POTENTIAL ROLE OF KALLIKREIN/KININ AS AN ANTIOXIDANT IN PROTECTION AGAINST CARDIOVASCULAR AND RENAL DISEASE AND STROKE

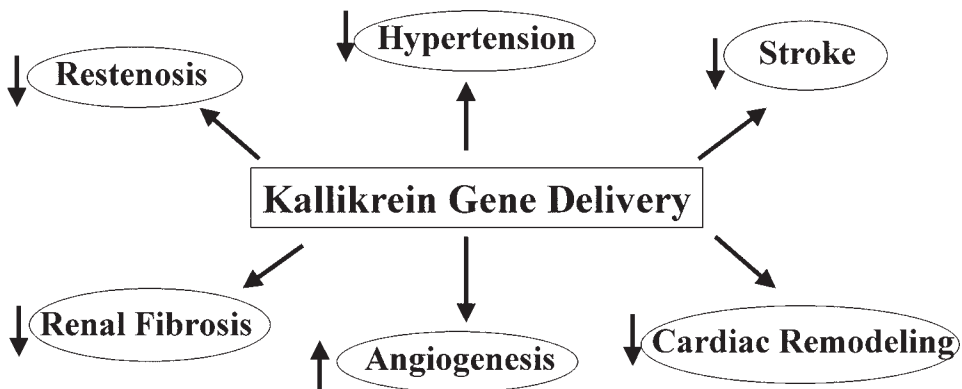
Reduced tissue kallikrein levels could result in reduced NO formation, as a result of lower kinin receptor activation. NO is a potent vasodilator and inhibitor of the formation of reactive oxygen species (87). Therefore, reduced NO bioavailability may lead to increased oxidative stress. Oxidative stress causes the activation of the signaling molecules and results in inflammation, apoptosis, proliferation, hypertrophy, and fibrosis eventually leading to cardiovascular and renal disease. Therefore, a continuous supply of tissue kallikrein by gene transfer or kallikrein protein infusion could reduce oxidative stress, leading to inhibition of inflammation, apoptosis, proliferation, and hypertrophy, and thus protect against cardiovascular and renal disease and stroke (Fig. 2).

### MULTIPLE ROLES OF KALLIKREIN/KININ IN CARDIOVASCULAR AND RENAL DISEASE AND STROKE

Using transgenic and somatic gene transfer approaches to achieve a continuous supply of kallikrein/kinin *in vivo*, we have shown that the KKS exhibits protective effects in hypertension and cardiovascular, renal, and central nervous systems. Our studies demonstrated that kallikrein gene delivery exhibits multiple beneficial effects in the heart, kidney, blood vessel, and brain in various animal models (Fig. 3). These include blood



**Fig. 2.** The protective role of tissue kallikrein as an antioxidant in cardiovascular and renal disease and stroke. NO, nitric oxide.



**Fig. 3.** Multiple functions of tissue kallikrein in the heart, blood vessel, and brain.

pressure reduction; attenuation of renal injury, cardiac infarction and cardiac remodeling; inhibition of neointimal formation in blood vessels after balloon angioplasty; and reduction of stroke-induced mortality, cerebral infarction, and neurological dysfunction. Enhanced kallikrein/kinin levels contribute to these pleiotropic effects by reducing apoptosis, inflammation, hypertrophy, fibrosis, and vascular smooth muscle and renal cell proliferation, and increasing angiogenesis (Table 1). Taken together, our results indicate that kallikrein gene transfer may have significant therapeutic potential for treating cardiovascular and renal diseases and stroke.



Table 1  
Pleitropic Effects of Kallikrein Gene Delivery on the Heart,  
Kidney, Blood Vessels, and Brain in Various Animal Models

<i>Organ</i>	<i>Effects</i>
Heart	↑ Cardiac function ↓ Hypertrophy ↓ Fibrosis ↑ Capillary density ↓ Apoptosis
Kidney	↑ Renal function ↓ Glomerular hypertrophy ↓ Fibrosis ↓ Cellular proliferation ↓ Apoptosis
Blood vessels	↓ VSMC proliferation ↓ Neointima formation ↑ Reendothelialization ↑ Angiogenesis
Brain	↓ Cerebral infarct size ↓ Cerebral hemorrhage ↓ Neurological deficits ↑ Glial cell migration and survival ↓ Apoptosis ↑ Angiogenesis

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## Current and Future Novel Targets of Gene Therapy for Hypertension

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### SUMMARY

Traditional therapeutic approaches for the treatment and control of hypertension are effective in normalizing blood pressure (BP) in less than a third of patients with hypertension. These pharmacological approaches may have reached a plateau in their effectiveness and newer strategies need to be investigated to not only increase the number of patients achieving BP control, but to find ways to cure the disease instead of just manage it. Since completion of the Human Genome Project and the continuous advancement of gene delivery systems, it is now possible to investigate genetic means for the treatment and possible cure for hypertension. In this review, we discuss potential genetic targeting for treatment of hypertension. There are two generalized gene transfer approaches that have been used successfully for hypertension. One is an induction approach where genes that lower blood pressure are overexpressed. A second method is a reduction approach where products of genes that are known to increase blood pressure are decreased. There are a variety of methods that have been utilized to meet these objectives, such as “knockout” and “knock-in” animal models, and the

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use of sense and antisense (AS) technology. This review will focus on the sense and antisense applications, and how this technique is becoming more refined and precise through the targeting of specific tissues, the regulation and induction of components of the system, and use of other newer technologies, such as short interfering RNA (siRNA). Our lab has generally focused on the reduction approach, specifically in the genetic manipulation of components of the renin–angiotensin system (RAS). This system not only modulates BP, but has also been implicated in cardiac hypertrophy and morphology and in insulin resistance, which is highly correlated with hypertension. We will also discuss how new genes can be identified and subsequently serve as targets for the treatment of human hypertension.

**Key Words:** Gene therapy; viral vectors; gene delivery; animal models; hypertension; renin–angiotensin system; gene arrays.

## INTRODUCTION

Hypertension is a chronic, debilitating disease that affects more than 50 million Americans (1,2). Although hypertension is defined as a condition of high blood pressure (BP) (1) (systolic BP  $\geq 140$  mmHg and diastolic BP  $\geq 90$  mmHg), it is a much more complex disorder, with a majority of hypertensive patients being insulin-resistant and/or salt sensitive (3–6). High BP is estimated to account for 6% of all deaths worldwide (7) and is the most common treatable risk factor for cardiovascular disease (CVD). A sustained elevation in BP contributes to serious health complications, such as myocardial ischemia and infarction, renal failure, stroke, and retinal damage (8–10). Vascular and cardiac remodeling occurs in hypertension, which has both hemodynamic and nonhemodynamic effects (11,12). This disorder has such a negative impact on health care that the most recent Joint National Committee on Prevention, Detection, Evaluation, and Treatment of High Blood Pressure report has suggested a new category of prehypertension in adults that have a systolic BP of 120–139 mmHg and a diastolic BP of 80–90 mmHg (13). The report states that individuals with this range of BP should be considered pre-hypertensive, and suggests health-promoting lifestyle changes to prevent CVDs.

Hypertension is an asymptomatic, chronic disease that is characterized as either primary or secondary. The latter is indicative of having a separate primary cause, whereas the primary (or essential) form of hypertension, which constitutes more than 90% of the cases, is of unknown etiology. This form of the disease is chronic, multifactorial, and multigenetic in origin, and is difficult to manage and currently not preventable or curable. Thus, the major goal of current therapies has been to control BP and prevent the complications (end-organ damage) associated with the disease, regardless of its origins. Despite the large arsenal of antihypertensive agents currently available, successful control of BP ( $\leq 140/90$  mmHg) is only observed in a small percentage of patients. Although only 29% of hypertensive patients in the United States have their BP controlled to the nationally recommended level of less than or equal to 140/90 mmHg with pharmacological intervention (13), the control rates are much worse in other countries around the world (14–16). Therefore proper treatment and management of hypertension is of critical importance to society and should be a matter of extreme urgency both in the United States and abroad.

## ADVANTAGE OF GENE THERAPY OVER CONVENTIONAL PHARMACOTHERAPY

Despite a large arsenal of therapeutic agents, conventional pharmacological therapy has not cured hypertension and, in fact, does not control the disease in the majority of

patients. Possible reasons for poor control of BP include incorrect targets of therapy, and poor education, economics, availability of health care, follow-up care, drug availability, and compliance. Even if the “ideal drug” was available for each patient, it is more likely that compliance would be the major factor in determining patient outcome. Reasons for a lack of compliance in hypertensive patients include convenience and side effects of the drugs utilized. Convenience can refer to the drug dosing regiment, which does not always conform to a patient’s everyday activities. Most pharmacological agents are short-acting ( $\leq 24$  h) and require multiple dosing per day. Another reason a patient may not stay on his/her medication is that symptoms related to the side effects of the drugs may be manifested in this otherwise asymptomatic disease. Patients can “feel better” when they are not on their medication, and they therefore either reduce their own dosage, or stop taking the medication altogether. Thus, we are at a stage in the treatment of hypertension where more focus must be placed on other treatment paradigms rather than further drug developments. Gene therapy offers the possibility of producing long-term effects with specificity based on the particular genetic target. Side effects may no longer be an issue, compliance would be nearly eliminated, as therapy can be directed at the source of the hypertension, and this therapy could be administered a minimal number of times over the patient’s lifetime.

## PHILOSOPHIES OF GENE THERAPY

Gene therapy promises to be the next frontier for treatment of and possibly a cure for complex diseases like hypertension. A variety of studies over the past few years have looked at different gene targets and gene delivery vectors as strategies. Currently, therapeutic agents are chosen by targeting the genes that are important in BP regulation. Whereas this technique should yield promising antihypertensive results, ideal gene targets would be those that have not only been implicated in hypertension, but also have been implicated in some of the target organ damage that is associated with hypertensive disease, such as the heart, kidney, vessels, etc. Over the last several years, our lab has mainly focused on the genetically manipulated renin–angiotensin system (RAS). This system has long been associated with hypertension. The RAS is essential in both the normal regulation of BP, as well as in the pathophysiology of hypertension. This system is also involved in, but not limited to, the alterations of nitric oxide metabolism (17), the impact of oxidative stress on endothelial function (18), vascular smooth muscle and cardiac hypertrophy (19,20), and insulin resistance (21,22). Additionally, there are numerous genetic link studies with components of the RAS and hypertension (23–30), as well as the successful use of both angiotensin-converting enzyme (ACE) inhibitors and angiotensin receptor blocking agents in the treatment of hypertension and some of its related complications.

There are two contrasting philosophies for gene therapy in hypertension—the reduction approach and the “induction” approach. In the “reduction” approach, genes that have been implicated in the elevation of BP are targeted, with the hope of reducing translation (in the case of a signaling molecule) or attenuating their effects by reducing their receptor’s population. With the induction approach, genes that have a BP-lowering effect are introduced or upregulated with selective gene and/or promoter delivery. That is, recombinant DNA is introduced in vivo to express mRNA and increase the concentration of the targeted protein. For each of these approaches there are a variety of methodologies utilized to produce the desired effects.



There are two major contrasting paradigms that can be used to produce these genetic alterations. One is the use of transgenic animals that knock out a specific gene or overexpress a particular gene. The other approach is to alter the genetic makeup of the animal after development with induction (sense) or reduction (antisense [AS]) approaches. A potential drawback to the knockout models is that the animal may undergo some altered physiology to compensate for the removal of a particular gene. This would be especially important if the gene that is removed has a major role in the developmental process. Although knockout models have made significant contributions, any compensatory changes that the animal undergoes may reduce the power of this type of strategy. Both strategies have been successful in reducing BP or in preventing the rise in BP in experimental animals; however, we are of the opinion that the genetic alterations should occur after the developmental process to avoid any compensatory physiological adaptations that may influence the interpretation of the significance of the particular gene of interest. Therefore, this review will focus more on the postdevelopment genetic manipulations in experimental hypertension.

### ***“Induction” Approach***

Several investigators have been successful in lowering BP using the induction paradigm. Overexpressing agents that are vasodilators, such as atrial natriuretic peptide (31–34), adrenomedullin (35–38) endothelial nitric oxide synthetase (eNOS) (39–42), human tissue kallikrein (43–55), superoxide dismutase (56–58), and the AT2 receptor (59), have been successful in reducing BP. These studies have examined different models of hypertension, and the gene transfer approach has been systemic or localized in specific tissue(s), depending on the gene target. Systemic delivery of these transgenes in plasmid DNA or adenovirus by intravenous, intraperitoneal, or intraportal vein injections results in a reduction of BP in a variety of animal models (*see* Table 1). Innovative delivery systems have been examined by a few research groups. Chen et al. (60) transfected Chinese hamster ovary (CHO) cells with atrial natriuretic peptide (ANP) cDNA, and then the CHO cells were encapsulated in nonantigenic biocompatible polycaprolactone capsules, prior to their implantation into the peritoneal cavity of rats. Li et al. (61) used encapsulated genetically engineered fibroblasts expressing ANP. In both cases, BP was significantly reduced in hypertensive Dahl and spontaneously hypertensive rats (SHR), respectively. In most examples of the induction gene transfer approach, transgenes were administered to adult animals and BP was reduced within a week and remained below controls for 4–6 wk. The extent of the reduction in BP was dependent on the dose of DNA injected, but averaged between 20 and 40 mmHg. Some of the differences observed regarding the extent and duration of BP effects may be related to the choice of promoter directing the expression of the transgene, the route of injection, and the vehicle in which the transgene is delivered. The “induction” gene transfer approach is not restricted to systemic administration. Gene transfer of eNOS, via an adenovirus vector, into the nucleus tractus solitarius of SHR also decreased BP (39,42). In all the studies undertaken, control viral injections were without effect, further demonstrating it was the transgene, and not the virus, that mediated the observed decreases in BP.

BP is not the only parameter assessed in these studies. Investigators have looked at cardiac and renal complications, which are summarized in Table 1. In addition, other parameters also have been evaluated. For instance, overexpression of human tissue kallikrein in a fructose-induced model of hypertension (43) not only lowered BP, but the

insulin-resistant state and the number of angiotensin type I receptors (AT<sub>1</sub>R) were also reduced. This gene transfer was without effect on control animals, which was the case in all the other gene transfer experiments described in Table 1. Other investigators (41,57) have also demonstrated that the impaired endothelial function observed in the SHR was significantly improved after gene transfer of eNOS and superoxide dismutase. The specificity of the responses to the transgene has been verified using agents that block or bind to the receptor that the upregulated protein would bind to (50,53).

### ***Reduction Approach***

Similar success in reducing BP and cardiovascular pathologies in experimental animals has been realized with the reduction gene transfer approach, which underexpresses agents that vasoconstrict vessels. The most successful agents used in this approach have targeted various components of the RAS. Table 2 summarizes the studies that have been utilized to reduce expression of various genes. These studies produce similar findings as described for the induction approach, as far as its range of effects on reducing basal BP or preventing the rise in BP in several different models of experimental hypertension. In many of these studies, administration of the AS transgene occurs during the neonatal stage and results in prevention of high BP in a variety of hypertensive models. Administration of AS to the AT<sub>1</sub>R in 5-d-old animals completely prevented the development of hypertension in the SHR (62–65). Reverse-transcription polymerase chain reaction (RT-PCR) and receptor-binding studies confirmed a 40–60% knockdown of the endogenous AT<sub>1</sub>R, thereby decreasing the gain of the RAS, but still leaving the system intact at a basal level (64,65). Responsiveness to angiotensin II (Ang II) is reduced in AT<sub>1</sub>R antisense-treated rats; however, BP is only reduced in hypertensive and not in normotensive animals. This would suggest that it is an overactivity of the RAS (as opposed to altered function) that is important in hypertension. As was found with the induction gene transfer studies, not only is BP reduced but so are many of the cardiac, renal, and vascular pathophysiologies normally associated with the hypertensive state. For instance, cardiac hypertrophy, fibrosis, perivascular necrosis in cardiac tissues, and endothelial dysfunction were prevented in SHRs treated with AT<sub>1</sub>R-AS delivered via a retroviral vector (62–65). Similar results were observed when AS to ACE was used as the transgene in a retroviral vector (66). Like the induction approach, there is also reversal of insulin-resistance in the fructose-model of hypertension with AS to the AT<sub>1</sub>R (67). Recent reports have suggested that the RAS is important in insulin resistance (22,68,69). This implicates an association of the RAS with insulin resistance and Syndrome X. Recent clinical and experimental evidence have demonstrated that antagonism of the RAS reduces the incidence and severity of diabetes and insulin resistance (70–72), providing further rationale for the use of components of the RAS as potential transgenes in gene therapy approaches to other chronic metabolic disorders.

Generally speaking, this reduction approach appears to produce effects on BP that are more long-lasting than the induction approach; however, this may be related more to the delivery system and its resulting integration of the AS into the genome.

When retroviral vector AT<sub>1</sub>R-AS was administered to adult SHR, we observed a transient but significant decrease in BP (73). This transient effect may be a result of the limited infection of nondividing cells by the retrovirus. Other investigators (74–78), using a viral delivery system that can infect nondividing cells, demonstrated that underexpressing genes of the RAS is effective in reducing BP in adult hypertensive rats.

Table 1  
Increased Gene Expression

<i>Transgene</i>	<i>Animal model</i>	<i>Delivery</i>	<i>Route</i>	<i>Effect on blood pressure</i>	<i>Comments</i>	<i>References</i>
Kallikrein	Fructose-induced SD SHR	Plasmid DNA	iv	Decreased	Decreased AT <sub>1</sub> R, decreased endothelin, normalized insulin	43
		Adenovirus	im	No effect	Increased capillary density in ischemic muscle	44
	5/6 reduction in renal mass	Adenovirus	iv	Decreased	Left-ventricular hypertrophy attenuated, protects against renal injury and cardiac remodeling	45
	Adult and newborn SHR	Adenovirus	im	Decreased		46
	Dahl salt-sensitive	Adenovirus	iv	Decreased	Reversed cardiac hypertrophy and fibrosis and renal damage	47
	5-wk-old Goldblatt—2	Adenovirus	iv	Delay of onset	Reduced left ventricular mass, protection from renal dysfunction	48
	Kidney/I Clip	Adenovirus	iv	Decreased	Decreased left ventricular hypertrophy, attenuated renal injury	49
	Dahl salt-sensitive	Adenovirus	iv	Delay of onset		50
	SHR	Oligonucleotide	sc	Delay of onset	Gender differences	51
	Adult and newborn SHR	Adenovirus	iv	Decreased		52
	SHR	Oligonucleotide	iv	Decreased	Decreased BP in adult but not in young SHR	53
	SHR	Plasmid DNA	im	Decreased	BP reduction attenuated with bradykinin antagonist	5
	SHR	Plasmid DNA	iv	Decreased	Hypotensive effect reversed by kallikrein inhibitor	55
Adrenomedullin	5-wk-old Goldblatt—2 Kidney/I Clip	Adenovirus	iv	Delay of onset	Decreased left ventricular mass, decreased myocyte diameter, decreased myocardial fibrosis, decreased renal	35

(continued)

	SHR	Plasmid DNA	iv	Decreased	2nd injection further reduced BP	38
Nitric oxide synthase	SHR	Adenovirus	icv (NTS)	Decreased	Depressor response in WKY, but greater in SHR	39
	Stroke-prone SHR	Adenovirus	iv	No effect	Improved endothelial function	40
	SHR	Plasmid DNA	iv	Decreased	2nd injection extended therapeutic time limit	41
	SHR	Adenovirus	icv (NTS)	Decreased	Decrease in HR and BP reversed by microinjection of soluble guanylate cyclase inhibitor	42
Atrial natriuretic peptide	SHR	Plasmid DNA	iv	Decreased	Increased urine volume and sodium excretion	31
	Dahl salt-sensitive	Adenovirus	iv	Decreased	Decrease in cerebral infarction, reduced thickness of arterial wall	32
	Dahl salt-sensitive	Adenovirus	iv	Decreased	Reduction in cardiac myocyte size, attenuation of glomerular sclerotic lesions	33
	4-wk-old SHR	Plasmid DNA	iv	Decreased	No effect on 12-wk-old SHR	34
Superoxide dismutase	SHR	Adenovirus	iv	Decreased	Effect greater in anesthetized compared with awake rats; improved endothelial function	56
	Stroke-prone SHR	Adenovirus	iv	No effect	Improved endothelial function	57
Heme oxygenase	5-d-old SHR	Retrovirus	ic	Delay of onset		58

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SHR, spontaneously hypertensive rats; iv, intravenous; im, intramuscular; ic, intracardiac; sc, subcutaneous; icv, intracerebroventricular; WKY, Wistar-Kyoto; BP, blood pressure; SD, Sprague-Dawley rats.

**Table 2**  
**Decreased Gene Expression**

<i>Transgene</i>	<i>Animal model</i>	<i>Delivery</i>	<i>Route</i>	<i>Effect on blood pressure</i>	<i>Comments</i>	<i>References</i>
Fibroblast growth factor	SHR	Liposome	iv	Acute decrease	Augments number of endothelial cells, ameliorated endothelial dependent response to vasoconstrictors	98
Angiotensinogen	SHR	Oligonucleotide	iv	Decrease	Plasma angiotensinogen and angiotensin II levels reduced	81
	SHR	Oligonucleotide	iv	Decrease	Angiotensinogen and angiotensin II levels reduced	82
	SHR	Oligonucleotide	icv	Decrease	Decreased AT <sub>1</sub> R in PVN, decreased angiotensin II in brainstem	83
	SHR	Oligonucleotide	icv	Decrease	Decreased angiotensin II in brainstem	84
	SHR	AAV*	ic	Decrease, delay of onset	Decreased left ventricular hypertrophy, decreased angiotensinogen	96
	SHR	Asialo-glycoprotein	iv	Decrease	Reduced hepatic angiotensinogen, reduced cardiac AT <sub>1</sub> R, decreased left-ventricular hypertrophy	97
	SHR	AAV	iv	Decrease	Greater effect of AAV-plasmid vector with liposome	100
	Cold-induced SD	Liposome	iv	Decrease	Decreased spontaneous drinking response	101
	Goldblatt—2 Kidney/1 Clip	Oligonucleotide	icv	Decrease	Decreased the elevated hypothalamus angiotensin II levels	105
Angiotensin converting enzyme	SHR	Retrovirus	ic	Decrease	No effect on WKY	66
AT <sub>1</sub> receptors	SHR	Retrovirus*	ic	Decrease	Decreased cardiac hypertrophy and fibrosis, similar effects seen in progeny	62
	SHR	Retrovirus*	ic	Decrease	Prevented left ventricular hypertrophy and myocardial perivascular fibrosis	63
	SHR	Retrovirus*	ic	Decrease	Decreased angiotensin II mediated responses in WKY and SHR but only decreased basal pressure in SHR	64
	SHR	Retrovirus*	ic	Decrease	Angiotensin II-induced BP and dipsogenic responses attenuated	65

Fructose-induced SD SHR	Retrovirus* Retrovirus	ic ic	Decrease Transient Decrease	Prevented glucose intolerance	67
SHR	AAV*	icv (Lat. Vent) icv ic	Decrease Decrease Decrease	Repeated daily injections for 6 d Intracardiac injection in 3-wk-old SHR also reduced BP	73 74
SHR L-NAME SD	Oligonucleotide Retrovirus*	icv ic	Decrease Decrease	Decreased left-ventricular hypertrophy, endothelial dysfunction unchanged	84 91
Renin transgenic Angiotensin-induced SD	Retrovirus*	ic	Decrease	Prevented cardiac hypertrophy	93
Cold-induced SD	Retrovirus*	ic	Decrease	Protected against angiotensin II-induced increases in BP and cardiac hypertrophy	99
Goldblatt - 2 Kidney / 1 Clip SHR	Oligonucleotide Oligonucleotide	ic and/or icv	Decrease Decrease	Spontaneous drinking response to cold reduced	101 102
Uni-neprectomized SD	Oligonucleotide Retrovirus*	i.v. intracisternally pump ic	Decrease Decrease Increase Increase	Decreased cerebral infarct after middle cerebral artery occlusion	104
SHR	Liposome	iv	Decrease	Increased pressor response to angiotensin II Increased pressor response to angiotensin II Weekly injections for 2 mo; Decreased left-ventricular hypertrophy, but only effective in 5-wk, not 13-wk-old SHR	79 80 92
Angiotensin-induced SD	Oligonucleotide	iv	Decrease	Normalized left-ventricular hypertrophy	108
SHR SHR	Liposome Liposome	iv iv	Decrease Decrease	No effect on heart rate Decreased beta 1, but not beta 2, receptors, decreased plasma renin activity and angiotensin II	90
CPY-like kininase	Oligonucleotide	iv	Decrease	Increased urine volume and sodium excretion	95 107
Tyrosine hydroxylase	Oligonucleotide	iv	Decrease	Decreased epinephrine and norepinephrine, decreased TH activity in adrenal medulla	94
CYP4A1	Liposome	iv	Decrease	Decreased sensitivity to constrictor action of phenylephrine	103
Thyrotropin	Oligonucleotide	it	Decrease		106

\*Injection performed at 5 d of age. SHR-, spontaneously hypertensive rats; AAV, adeno-associated virus; WKY, Wistar-Kyoto; it, intrathecal; iv, intravenous; icv, intracerebroventricular; ic, intracardiac.

Further, there have been some recent studies (79,80) that have used gene therapy approaches to lower expression of the AT<sub>2</sub> receptor, which results in the increase in basal BP. Collectively, these studies indicate that both induction and reduction of components of the RAS are promising for the treatment of hypertension.

## DELIVERY METHODS FOR GENE THERAPY

In hypertension, whether using an AS approach to express reduction or AS mRNA to inhibit an overexpressed protein, or using an induction or sense approach to increase the synthesis of proteins critical to the pathogenesis of the disease, one generally has to transduce somatic cells of the body. Therefore, methodologies for efficient gene transfer are paramount for successful gene therapy. Other essential components of successful gene therapy are the ability to appropriately express the gene, to have long-term survival of the transduced cell(s), and to be able to control expression of the particular gene. There have been several vehicles explored for somatic gene delivery for hypertension, such as naked DNA, liposomes, receptor-mediated delivery, and viral delivery. This last method can be further divided by the type of virus utilized: retroviral, adenoviral, adeno-associated, or lentivirus. Each system has certain advantages and disadvantages, which influence their selection. The duration of the effect of the target gene is dependent on the delivery system utilized. The following is a brief summary of some of the more common gene delivery vehicles.

### *Nonviral Vectors*

Nonviral vectors possess some advantages over viral vectors for gene therapy, in that these vectors are usually devoid of the safety issues related to insertional mutagenesis and immunogenicity. Early attempts to modify genes to lower BP used oligodeoxynucleotides (ODN). In particular, AS-ODN was directed to the AT<sub>1</sub>R and angiotensinogen mRNA (81–84). ODN are single-stranded, short sequences of nucleotides that are made of DNA (or modified DNA) designed to interact specifically with its target mRNA by Watson–Crick basepairing. Once bound to its target mRNA, the AS-ODN inhibits protein synthesis by directly blocking translation (85,86). ODNs may further work to block expression by stimulating RNase H, which sterically inhibits the mRNA from translating its message. This reduces the total number of mRNA copies and frees the AS-ODNs to hybridize again. The ODN approach has some specific disadvantages (77,80–87). Its effects are long-lasting in comparison to available pharmaceutical agents, but usually the effects are still limited a week after administration. No adverse or toxic effects have been observed, they can be produced in larger quantities relatively inexpensively, and they do not cross the blood–brain barrier when given peripherally (80). There have been numerous studies conducted, mostly in the SHR, with BP generally reduced anywhere from 16 to 40 mmHg for 3–7 d (*see* Tables 1 and 2). Naked AS-ODN is effective, but cationic liposomal carriers (77,88–90) can increase the effectiveness.

Naked DNA can be delivered directly to the cells, in a plasmid (circular, naked DNA) or with liposomes (lipid bilayers that carry the DNA into cells). These methods are relatively safe and easy. Plasmids are effective vectors, but administer relatively short transgene duration because they do not allow for integration into the genome. Human kallikrein and NOS have been manipulated using these techniques to transiently lower BP in hypertensive rats (*see* Table 1). However, these methods are not very efficient, may be cytotoxic, and only produce a transient effect (109).

Modifications to nonviral vectors can increase the efficiency of gene transfer to cardiovascular tissues. Liposomes developed with cationic lipids can allow for a higher transfection efficiency of plasmid DNA. Another method to deliver DNA is a receptor-mediated method using hemagglutinating virus of Japan liposome. In this method, DNA is combined with a ligand to be internalized by a cell in a specific receptor-mediated mechanism (110). Although this method is an improvement over the basic liposomal method and is highly specific, it is more difficult to produce the modified liposomes.

Recent advances in nonviral delivery technology have infused renewed vigor into the field. These newer advances include ultrasound-mediated enhancement of gene delivery and peptide-targeted liposomal formulations to target enhancement. With regard to cardiovascular physiology, ultrasound has recently been shown to enhance transduction into vascular tissue by increasing permeability of the cell membrane. Lawrie et al. (111) has reported a 300-fold increase in gene transfer in smooth muscle cells compared with naked DNA alone using an ultrasound approach. Use of a lipid integrin DNA (LID) nonviral vector system, which is a modification of the liposomal-mediated gene delivery, consists of a standard liposomal/DNA component that is complexed with an integrin-targeting peptide. This LID vector complex can be used to attach to cell receptors on vascular tissues. This targeting has been used effectively *in vitro* for both vascular smooth muscles and endothelial cells (112). This technology can be a versatile one in which substitution of other peptides that can mediate selective uptake into specific cell types can be developed to enhance targeted gene delivery. If more prolonged effects are desired, the delivery of the transgene requires a viral vector. There are numerous viral vectors used for gene therapy, each having its specific advantages and disadvantages (Table 3).

### ***Viral Vectors for Gene Therapy Approach***

For hypertension, where a long-term (or permanent) control of pathophysiology is the goal, integration of the transgene into the host cell's genome is necessary. Whereas naked DNA and liposomes are incapable of this task, viruses require such actions for their life cycle. Various viral vectors have been modified and used to deliver and incorporate transgenes of interest into cells. There are four major classes of viral vectors currently being used for both *in vivo* and *in vitro* applications in hypertension (Table 3). However, the "ideal viral vector" has yet to be found. An ideal vector should be safe (have a low toxicity when delivered), should not elicit an immune response, should integrate in a predictable, safe region of the genome, must be efficiently taken up by the target tissue, and must infect the target tissue with a high enough efficiency to have a physiological effect. For practical purposes, the vector also has to be packaged with high efficiency into the viral envelope, be easily produced in high concentrations, and have enough space to accommodate the required transgene and its promoter. Each of the known viral vectors has some of these characteristics, but not all of them.

### ***Adenovirus***

Adenovirus is a double-stranded DNA virus that can infect both dividing and nondividing cells with high efficiency as it targets mammalian cells with specific membrane receptors. Viruses then enter the cells by receptor-mediated endocytosis and translocate into the nucleus. Most of the adenoviruses are episomal, and thus do not integrate into the host DNA. Because they are not integrated, the episomal DNA eventually becomes inactive or degraded, and the transgene will no longer be expressed. As the adenoviral genome



Table 3  
Major Pros and Cons of Delivery Methods

<i>Vehicle</i>	<i>Pros</i>	<i>Cons</i>
Oligonucleotide	Easy to produce, safe to handle	Lack of target specificity, Transient expression patterns
Liposome	Easy to produce, safe to handle	Lack of target specificity, Transient expression patterns
Viruses		
Adenovirus	Infects dividing and nondividing cells	Immunogenic, transient expression, random integration
AAV	Nonpathogenic, specific integration site (wild-type), infects nondividing cells	Very small payload
Retrovirus	Stable long-term expression, large payload	Random integration, only infects dividing cells
Lentivirus	Stable long-term expression, large payload, infects dividing and nondividing cells	Random integration

AAV, adeno-associated virus.

expresses numerous viral proteins, the adenovirus stimulates the immune system and can cause vascular inflammation (113,114). Utilization of this vector system has been shown to delay the development of hypertension in genetic and nongenetic models of hypertension for approx 1 mo (see Tables 1 and 2). Because of the transient expression patterns and induction of the immune response, the conventional forms of the vector have serious limitations and will likely not be used for human gene therapy for chronic diseases. With advances in virology, however, use of this vector may prove beneficial in the future.

Recent advances in engineering retroviral vectors have increased transduction of cardiovascular cells. Two methods shown to improve gene delivery into vascular beds are serotype switching and the use of antibodies. The adenovirus family is very diverse and there have been over 50 different serotypes identified. Pseudotyping technology can exploit certain serotypes to target gene delivery to specific target tissues. For CVDs, utilization of this approach has been successful in improving gene delivery. For example, serotypes 4 and 11 possess higher affinity binding and infectivity for vascular endothelial cells (115). Harari et al. (116) reported that they were able to target activated endothelium in vitro by using an antibody-mediated adenovirus directed against e-selectin. This concept may allow for strategies to target specific vascular beds in vivo.

### ***Adeno-Associated Virus***

Adeno-associated virus (AAV) is a stable, nonpathogenic vector that can infect nondividing cells. The AAV is a parvovirus, and its replication is dependent on the presence of a helper virus. The wild-type AAV integrates in a specific region of chromosome 19. Although its site of integration is known, the recombinant AAV (rAAV) loses this property and randomly integrates. There are some limitations with this vector system because it is difficult to produce large quantities of virus and it has a limited transgene carrying capacity (117–119). Although the carrying capacity is limited, this virus is amply suited

for the delivery of small genes and AS cDNA, which only needs to code for a small fragment of the target gene to be effective. This virus is also fairly safe for human use. AAV is not pathogenic and is not associated with any known disease state.

To produce rAAV a helper virus (adenovirus) is required. In producing the rAAV, viral coding sequences are removed so that the source of immune reactions to viral gene expression is removed and no inflammation response is evoked *in vivo*. AAV infects all mammalian cells, and its expression in target tissue is long-lasting (118). With no helper virus present, AAV infection remains latent indefinitely. Upon infection of the cell with helper virus, the AAV genome is excised, replicated, packaged, and finally released by the cell.

The AAV is a good candidate for gene therapy because it is safe and has a very broad host range. AAV has been successfully used in phase 1 clinical trials for the treatment of cystic fibrosis (120). It has also been used to deliver AS to the AT<sub>1</sub>R in hypertensive rat models. Use of rAAV containing AT<sub>1</sub>R-AS has been administered systemically as well as centrally in the SHR (74), and resulted in a decrease in BP and a reduction in left-ventricular hypertrophy.

### ***Retroviral Vectors***

Retroviral vectors, which have a large transgene payload capacity, can be produced with high efficiency and titer. The viral genome is a single-stranded RNA that is converted to DNA in the host cell by reverse transcription mediated by viral reverse transcriptase. The DNA is then integrated into the genome and can result in long-term expression of the transgene. This virus type is highly efficient at delivering genes to dividing cells; however, this vector is not efficient at infecting nondividing cells. The other major disadvantage of retroviruses lies in their random genome integration pattern (121), which raises concerns about their safety for practical use *in vivo*. Despite this disadvantage, we and others have been very successful in using the retrovirus to prevent hypertension, cardiac hypertrophy, and restenosis in several experimental models (62–67,91,93,99,122).

### ***Lentivirus***

The lentiviral vectors are a subfamily of retrovirus and are the most recent addition of vectors to the rapidly developing field. The lentiviral vectors are derived from the human immunodeficiency virus type 1 (HIV-1). These highly “humanized” vectors combine the advantages of retroviral and adenoviral vectors, and they are emerging as the vectors of choice for long-term, stable *in vitro* and *in vivo* gene transfer. These vectors are attractive because they can efficiently carry large transgene cassettes (up to 18 kb in size) and they are capable of transducing both dividing and quiescent cells. Lentiviruses can allow for long-term expression of the transgene with little immune response. Lentiviruses can infect noncycling and postmitotic cells and have the potential to generate transgenic mice by infecting stem cells (123). This vector has been successfully used to transduce genes into selected tissues of adult animals with a long-term expression potential (124). However, its use *in vivo* has been limited by production. We have recently developed an efficient method for packaging and concentrating lentiviral vectors that consistently yields high-titer virus on a scale suitable for *in vivo* applications (125). We demonstrated that lentiviral vector delivered systemically can transduce several cardiovascular-relevant tissues, including the brain, and the transgene exhibited long-term (120 d, duration

of experiment) expression (126). Recent findings from our lab using this lentivirus to overexpress the AT<sub>2</sub>R in SHR demonstrated that our transgene effectively transduced cardiac myocytes and significantly reduced the cardiac hypertrophy in these animals without effecting BP (59). These findings provide significant information with respect to the AT<sub>2</sub>R and cardiac hypertrophy, and suggest that the AT<sub>2</sub>R do antagonize effects mediated by the AT<sub>1</sub>R.

The lentivirus preparations we used were administered to 5-d-old animals. Future studies will determine the effectiveness of utilizing this vector delivery method in adults. Because the lentivirus can infect nondividing cells, it should be effective in adult animals and is most likely more applicable in a clinical setting over the retrovirus, because it could reverse the disease state in contrast to preventing the development of the disease.

### CELL TYPE-SPECIFIC PROMOTERS

Numerous technological advances have afforded us the ability to underexpress (reduction approach) or overexpress (induction approach) transgenes of interest in various animal models. However, ubiquitous expression of a transgene may lead to undesirable effects. Local or targeted gene delivery has the advantage of concentrating the gene therapy in a relevant tissue, and reduces the risk of losing the vector to sites where its under- or overexpression may have deleterious consequences. One way to obviate this potential problem is to utilize tissue- or cell-selective promoters to limit transgene expression to a subset of cells or tissues. Such systems have been developed to selectively alter gene expression in the heart and vascular smooth muscle (127,128). For example, tissue-specific gene expression can be enhanced by the use of specific cardiac myocytes promoters, such as *mlc-v* (ventricular-specific myosin light chain-2), or cardiac troponin T gene promoter (129,130). Others have used a rat neuron-specific enolase promoter (Ad-NSE) to specifically target neuronal cells (131). Thus, one can selectively target gene delivery to specific tissue types, provided one has utilized the appropriate tissue-specific promoter. Utilization with these specific promoters will limit expression of the transgene to specific tissue sites that are relevant for the particular disease state.

Targeting specific tissues/cells with a transgene early in development can have detrimental or even lethal effects. For example, transgenic mice with endothelially expressed genes have been reported to harbor severe developmental abnormalities (132). Thus, the more optimal approach to gene therapy is the development of system(s) that allow for externally regulated control of transgene expression.

### REGULATABLE PROMOTERS

Constitutive expression of a transgene may produce undesirable effects if nonphysiological levels of proteins are produced. Although gene therapy approaches have extraordinary promise, uncontrolled transgene expression may lead to deleterious consequences. Therefore, the ability to modulate transcription of a transgene or vector would be desirable for effective and safe gene therapy in humans. One way of overcoming this problem is to utilize a regulated gene expression system that uses exogenous ligands to control transgene expression. Some of these previously developed systems use inducible promoters, which are modulated by exogenous ligands, such as mifepristone (133), rapamycin (134), ecdysone (135), or tetracycline (136), to drive the expression of the transgene. These systems can allow for the activation of a transgene when needed, thereby

allowing researchers and clinicians to “turn off” of the transgene expression if complications arise, or to “turn on” the transgene when it is needed. In general, these systems use an inducer or repressor that can reversibly bind the endogenous ligand, and its chimeric state then acts as a transcriptional factor for its particular promoter. Ideally, such an exogenous inducer would allow a wide therapeutic window and few, if any, side effects when compared to conventional pharmacological therapy.

Pioneering work by Bujard and Gossen (137) established the tetracycline transactivator system as a reliable tool for regulating transgene expression. This tetracycline (*tet*)-inducible system consists of three major components: the tetracycline-binding-transcriptional modulator protein, the corresponding tetracycline-responsive promoter element and the tetracycline-class pharmacological agent. Doxycycline (Dox) is the most commonly used tetracycline drug used with the *tet* system because it has a high affinity for the *tet* transcriptional modulator protein, has a low toxicity, and favorable pharmacokinetic properties for use in vivo (138,139). This system has been used for transcriptional control of transgenes for over 10 yr, and a continuous line of modifications to the system have made it much more effective in tightly regulating gene expression (140).

The original *tet* system was a “*tet-off*” version, in which application of a tetracycline caused attenuated expression of the transgene. Subsequent development of the *tet* system by Gossen et al. (136) resulted in the generation of a “*tet-on*” version, where application of a tetracycline causes increased transgene expression. Despite recent improvements in the *tet-on* system, including the addition of a *tet*-silencer protein (141), this inducible system has an inherent leak in basal transgene expression and causes only mild increases in transgene expression (when compared with the range of expression of the *tet-off* system) (142). Certain other problems with the *tet-on* system are currently being worked out, such as cellular toxicity, insensitivity to Dox in certain tissues, and unstable transcripts (143,144). Some groups (141,145), however, have suggested that differences in regulation may exist among various mouse strains.

A possible drawback with this system is that it requires two vectors, and these vectors both have to infect the same cell to achieve a properly regulated system. Despite this issue, our lab constructed dual cassette retrovirus vectors that together encoded a modified enhanced green fluorescent protein (d2EGFP) under the control *tet-on* system. Viral particles were then used to infect rat aortic vascular smooth muscle cells and pulmonary endothelial cells. Cells transduced with both vectors were examined by fluorescence microscopy for inducible expression of d2EGFP. Incubation of dual-vector infected cells with Dox caused a robust expression of d2EGFP within 48 h, whereas removal of Dox caused a disappearance of green fluorescence within 24 h, (146). These results demonstrate tightly regulated gene expression using a retroviral vector system. We subsequently used this system to effectively induce AT<sub>1</sub>R-AS, resulting in a lowering of BP in the SHR (147) when the animals were maintained on Dox.

However, with newer technologies, these regulatable systems will have even greater advantages. For instance, Teng et al. (148) have recently combined the components of the regulated system with tissue-specific properties to direct the controlled induction of an exogenous transgene to the vascular endothelium of an adult mouse by utilizing an endothelial cell-specific promoter through the *tet-on* system.

Recently some *tet*-regulated transgenic mice have been developed. This reduces the payload requirement of a gene therapy vector in these animals, because the animals already harbor half of the gene-switch system ubiquitously. Corbel and Rossi (140) recently

summarized work on new tissue-specific tetracycline (activation and repression) transgenic mice, as well as the recent methods utilized to deliver tetracycline-regulated genes *in vitro* and *in vivo*. Ju et al. (149) recently generated transgenic mice with the use of an arterial smooth muscle cell (SMC)-restricted (SM22 $\alpha$  promoter-driven), tetracycline-controlled transactivator (tTA) to effect conditional expression of a tTA-dependent transgene encoding rat vascular chymase (RVCH). The recombinant RVCH converts Ang I to Ang II *in vitro*. In their study, hypertension was completely reversed and the medial thickening of mesenteric arteries from tTA<sup>+</sup>/RVCH<sup>+</sup> mice was prevented by doxycycline. Therefore, by utilizing such combined technologies, more specific and controlled targeting of selected transgenes can be employed more effectively as therapeutic tools.

More recently, use of endogenous instead of exogenous regulatable systems have been used effectively *in vivo*, further demonstrating the importance and versatility of effectively controlling gene expression and its application to hypertensive therapy. Kantachuesiri et al. (150) have used a rat CPY1a1 enhancer/promoter sequence to control mouse Ren2 cDNA expression in rats to create an inducible model of hypertension. BP and the RAS returned to normal in the absence of the inducer. Other *in vivo* work has used adenovirally mediated transfer of ecdysone-inducible constructs to the heart and carotid bodies of rats (151). Thus, these regulated systems offer significant advantages in utilizing gene therapy approaches to better understand disease processes and provide potential therapy for a variety of disease states.

Another example of an endogenous state-specific promoter is the hypoxia response element (HRE), which responds to hypoxic conditions within the cell. Under hypoxia, the transcription of numerous genes can be activated, including erythropoietin (152,153), the  $\beta$ -adrenergic receptor (154), glycolytic enzymes (155,156), and vascular endothelial growth factor (157,158), among others. Thus, this promoter could be used in various cardiovascular or metabolic disorders to regulate expression of genes that have been proven to have beneficial effects in conditions of ischemia, such as AS to the AT<sub>1</sub>R (159), the  $\beta$ -adrenergic receptor (160,161), superoxide dismutase (162,163), angiotensin-converting enzyme (164,165), and vascular endothelial growth factor (166).

Vigna et al. (167) were the first to report the generation of lentiviral vectors capable of delivering *tet*-regulated gene delivery to human hematopoietic progenitor cells *in vivo*. The combination of lentiviral delivery with improved transcriptional activators of the *tet* system resulted in the regulation of an inducible gene that was maintained for over 20 wk after infection of these engineered cells. The lentivirus, with its much larger cassette site, may allow for both the tetracycline transactivator and the tetracycline response element to be housed in a single vector. We have used such a system for *in vitro* manipulation and are currently attempting to develop such a vector for subsequent *in vivo* gene therapy for hypertension in order to better control expression of our transgene of interest. Thus, these regulated systems offer significant advantages for the utilization of gene therapy approaches to better understand disease processes and to provide effective therapy for a variety of disease states.

### ***CRE/Lox System***

Another type of regulated system is an older CRE (“causes recombination”)/Lox system. This technique allows for the modification of a specific gene with locus of crossover (loxP) sites flanking the specific region of interest through the use of standard

gene targeting vectors in embryonic stem cells. These targeted alleles are said to be “floxed”, or flanked by the loxP, and thus may not be fully functional. CRE recombinase directs recombination between loxP sites. Application of this technique in mice allows for deletion of selected material at specific times. This offers advantages over knockout models in that it can avoid the complications of not only embryonic lethality, but also the developmental compensatory changes that occur when there is a genetic knockout. Because this technology relies on homologous recombination in embryonic stem cells, its *in vivo* use is currently restricted to mice. Mice that are derived from these targeted embryonic stem cells are then bred to homozygosity for the targeted floxed allele and can then be crossed with other mice transgenic for CRE recombinants under the control of specific promoters to allow for a tissue-specific deletion of the floxed segment. Thus, the activity of a particular gene can be modified in a limited number of cells or tissues while the genetic content of the rest of the animal is essentially unaltered. Many tissue-specific CRE transgenic mice strains are becoming available and have provided insights into endocrine physiology in recent years. Most of these tissue-specific CRE/loxP-mediated gene deletions have been studied in endocrine or other target tissues, and have been nicely summarized by Ryding et al. (168). A database cataloging these mouse models is available at <http://www.mshri.on.ca/nagy/cre.htm>.

Unfortunately, most CRE/loxP mouse experiments have several potential pitfalls. First, expression of CRE within a particular tissue or cell is rarely uniform, which can lead to mosaic recombination. A second potential pitfall is that of transcriptional interference by vector-derived sequences. Like strain differences observed with the *tet* system (141,169), one might also anticipate that the efficiency of CRE recombination may also be strain-dependent, although there has yet to be any formal investigation of this possibility. Despite these potential drawbacks, the CRE/loxP technology is a well established genetic tool for the study of conditional deletion of genes in mice.

### ***Vigilant Vector Approach***

Recently, some groups have begun work on developing cell-state-specific promoter systems for transgenes. A popular example of this methodology is referred to as the “vigilant vector,” which senses hypoxic conditions within the cell and responds by increasing transgene transcription. This approach has been used to signal dormant transgenes to become active and to protect specific tissues with high amplification of the specific transgene. In order for this system to be effective, four components are required (170). The first component is stable vector that is safe and can be administered by systemic injection and in which the transgene is expressed in a particular organ or tissue. The second component is a reversible gene switch that acts as a biosensor and can detect certain physiological signals. The third component is a tissue-specific promoter, and the fourth component is an amplification system. This type of system can have broad applications. One can switch tissue-specific promoters or switch out different transgenes and/or protective genes in order to apply this vigilant approach in a variety of disease states. Tang et al. (171) recently characterized a hypoxic inducible double plasmid system for myocardial ischemia. This hypoxia-sensitive promoter could be used to drive transcription of hypoxia-protective genes, such as superoxide dismutase (162), the  $\beta$ -adrenergic receptor (160), and components of the RAS (159,164), thus conferring some immunity to hypoxia upon these cells.

### *siRNA Technology*

Selective downregulation (knockdown) of a specific gene can be a useful method for therapeutic intervention, provided the targeted gene is important in the pathogenesis of the disease. One way to facilitate this genetic “knockdown” is the use of AS gene technology. The major disadvantage of this method is that the transgene product (the AS mRNA) is destroyed along with the target mRNA. A recent advancement to the AS approach is a conserved posttranscriptional gene silencing (PTGS) mechanism mediated by double-stranded RNA (dsRNA). Silencing of gene expression using dsRNA, known as RNA interference (RNAi) or short interfering RNAs (siRNAs), provides a powerful tool for analyzing gene function. Although not all of the mechanisms by which this system operates are completely known, this RNAi method is more efficient than AS in that it is able to cleave the target mRNA and mark future copies of the target mRNA to be destroyed, while the interfering mRNA itself is not destroyed along with the target RNA (172). It can therefore cleave and destroy multiple pieces of target RNA. An RNase III-like enzyme called a “Dicer” cuts long dsRNA, or hairpin RNAs, into double-stranded siRNAs (173). These are typically 20–25 nucleotides (nt) long, and can trigger formation of an RNA silencing complex (RISC). The siRNA–RISC complex seeks out the mRNA with the targeted sequence and degrades it, effectively silencing that gene. Short dsRNAs can be synthesized *in vitro* and introduced into mammalian cells. Using these short siRNAs does not trigger antiviral mechanisms within the cell and can mediate gene-specific suppression in mammalian cells. As a result, a few copies of the interfering dsRNA can cause total degradation of cognate transcripts in a cell. This technology has been used successfully as a tool to analyze gene function in plants, insects, and nematodes (174–176); however, use of long dsRNA in mammalian somatic cells results in activation of antiviral defense systems that can result in nonspecific degradation of RNA transcripts and a general loss of host cell protein synthesis, thus investigators soon discovered that introduction of smaller siRNA, less than 30 nt, can lead to gene specific silencing (177).

Although there is significant potential for the use of this new AS technology to target specific genes associated with hypertension, important issues remain unresolved. Because there is no set pattern or rule yet discovered to determine the optimal sequence to target within a gene, investigators are encouraged to select several siRNA sequences in different locations along the gene of interest to test in order to find the most efficient site that will be important in silencing the gene of interest. A single-point mutation in certain locations of the paired region of the siRNA duplex can abolish target mRNA degradation (177). siRNAs are extraordinarily effective at lowering the amounts of targeted RNA—and, by extension, proteins—frequently to undetectable levels. However, any polymorphism in the gene of interest can complicate the process. There are several web-based tools to assist in design of siRNAs (examples include those provided by Ambion [<http://www.ambion.com>] and Dharmacon [<http://www.dharmacon.com>]). Once a sequence is identified, the siRNA transgene must be chemically synthesized and transfected. Recent evidence suggests that the effectiveness of siRNAs may depend greatly on the method of transfection (178). There is no uptake of siRNAs into cells, and the delivery to selected sites of therapy remains problematic (179). Handling of the RNAi is laborious and complex because of ubiquitous RNases. However, with advancing technologies, this may be a very effective means to knock down targeted disease-producing genes. Various viral vectors are being evaluated to increase infection for *in vivo* administration of siRNA.

Lentiviral and other retroviral systems have been shown to be effective in many settings and adenovirally based vectors are also being evaluated (180,181). The ability to efficiently and stably produce and deliver sufficient amounts of siRNA to the proper target tissues still requires refinement before this new technology can be tried clinically. Xia et al. (246) recently described a virally mediated delivery mechanism that results in specific silencing of targeted genes through expression of siRNA. These investigators demonstrated expression of exogenous and endogenous genes in vitro and in vivo in brain and liver, and further applied this strategy to a model system of a major class of neurodegenerative disorders, finally suggesting that this virally mediated strategy could be successfully used to reduce expression of target genes to provide therapy for human diseases. Lewis et al. (182) recently described a method for efficient in vivo delivery of siRNAs to organs of postnatal mice. Lentivirus-based vectors may be the most promising siRNA expression system to drive gene inhibition in stem cells, because the transgenes that are expressed from the lentiviruses are not silenced during the developmental process and can thus be used to generate transgenic animals through infection of embryos (180,183). Cheng et al. (184) recently reviewed this field, and provide an overview of its potential applications in the treatment of human disease.

## MICROARRAY TECHNOLOGY AND ITS IMPLICATIONS IN GENE THERAPY

Recent advances in genomic techniques and their integration into physiology have resulted in the genesis of the “functional genomics” era. Functional genomics is a multidisciplinary approach to studying genes, their products, and interactions among them that are responsible for mediating physiological responses. Also included in functional genomics is the detection and characterization of aberrations of genetic processes that may result in diseases. Recent advances in molecular biology and technology have made it possible to monitor the expression level of numerous genes simultaneously. Cardiovascular research has focused mainly on only a small fraction of known genes; however, information gleaned from genome sequencing and the development of microarray technologies has the potential to provide for genome-wide analysis of genes that can mediate BP regulation and their potential contribution to the pathogenesis of hypertension. Protein microarrays also have been developed for protein expression analysis and interaction analysis, which may include protein–protein, ligand–receptor, enzyme–substrate, and nucleic acid–protein interactions. However, the use of high-throughput arrays (both RNA and protein) has yet to realize its full potential in cardiovascular research. Issues concerning reproducibility and preanalytic variables can make the discovery process from these methods tedious (185,186).

Numerous investigators are using gene expression profiles in hypertensive models in hopes of identifying genes relevant to hypertension. Candidate genes can be identified from transcript profiling, and different strategies can be utilized to evaluate their potential relevance in the pathogenesis of hypertension. Clinical studies have demonstrated that genetic variation accounts for up to half of the phenotypic variation in BP (187). Numerous studies have demonstrated links between genes of the RAS (i.e., *angiotensinogen*, *renin*, *ACE*, and *AT<sub>1</sub>R*) and hypertension in various populations (188,189). The use of inbred rat models of hypertension and congenic strains also has identified large chromosomal regions containing quantitative trait loci that account for genetic variation in BP



(190–193). The construction of minimal congenic strains has been used to further narrow down the particular loci involved (194). However, consistent associations have been difficult to demonstrate, and little progress has been made toward identifying the specific genetic variants that contribute to a particular phenotype. Therefore, to better understand a polygenic disease such as hypertension, we need to identify which groups of genes account for the hypertensive phenotype, and how these gene clusters are regulated.

Gene expression profiling has been utilized to gain insight into disease mechanisms. Assessment of the expression of a large number of genes have been realized by the use of high-throughput gene profiling technology, such as cDNA and oligonucleotide microarrays, as well as serial analysis of gene expression (SAGE). In addition, for genomes that are not yet fully sequenced, transcript profiling of expressed sequence tags (EST) provides expression information on novel genes. The technical limitations and issues relating to analysis of microarray data have been reviewed recently (195–198). Gene expression profiles most often represent complex phenotypes. Cluster analysis of expression data may distinguish particular groups of genes that are regulated in a similar manner. Profiling has been used to identify genes involved in hypertension, and usually compares differentially expressed genes in inbred genetic rat models of hypertension with their normotensive controls. Differential gene expression between these two groups, however, may not necessarily reflect causative mechanisms in hypertension. The differences may reflect a genetic difference between the strains that are unrelated to hypertension, or they may be expressed as a result of the high BP and not be related to any cause for the increase in BP. In addition, like any method, there are some technical drawbacks for the sole use of microarrays in identifying candidate genes. Some limitations and issues relating to analysis of microarray data have been reviewed recently (196,197,199,200). Therefore, it is essential that other techniques, such as QTL mapping, complementation testing, or loss/gain of function experiments, be combined with the gene profiling to determine if differential gene expression reflects a causative role in hypertension (201–203).

Transcript profiling of inbred genetic models of hypertension, such as the SHR, have proven useful in identifying potential genetic mechanisms of hypertension. Microarray profiling from kidneys of SHR and Wistar–Kyoto (WKY) animals has identified a number of differentially expressed genes in SHR (203,204). However, even when comparing different strains of SHR, there is a large variability in genes that are differentially expressed, which underscores the genetic heterogeneity of the different strains (203). These observations suggest that there can be both common and distinct genetic mechanisms in hypertension in different SHR strains.

The most appropriate tissue type for gene profiling is debatable. Most transcript profiling studies in hypertensive rats have used RNA derived from kidneys (202,203) to identify genes that may contribute to hypertension, as the kidney has a well-established role in long-term regulation of BP. We are currently utilizing gene expression profiling in different areas of the brain, such as the hypothalamus and brainstem, in order to identify target candidate genes that may be regulated by the brain RAS in the SHR (205,206). To eliminate any genetic alteration resulting secondarily from an elevation in BP, we have also assessed gene expression profiles in primary neuronal cultures derived from the hypothalamus and brainstem areas of neonatal (prehypertensive) SHR and WKY rats (205). We found that neonatal SHR neurons in culture exhibit increased AT<sub>1</sub>R transcript expression and functional receptors as compared to WKY cultures. These results indicate that the hyperactivity of the brain RAS observed in the adult SHR is also present in the

neuronal cultures (207). We identified genes that are regulated by Ang II by comparing transcript profiles of neuronal cultures treated with vehicle to those treated with Ang II. Utilizing gene filtering and statistical analysis, we identified differential expression of 299 genes and 109 EST between strains (205). Interestingly, SHR neurons exhibit an altered Ang II-induced pattern of gene expression as most differentially expressed genes were upregulated, whereas their expression was downregulated in WKY neurons (a complete list is available online at [www.med.ufl.edu/phys/raizada/veerasingham.doc](http://www.med.ufl.edu/phys/raizada/veerasingham.doc)). This effort, like most of the gene expression profiling, has been of a correlational or descriptive nature. Further experiments are required to evaluate the physiological relevance of the altered gene profile of Ang II-responsive genes in SHR neurons, as well as that of genes that are differentially expressed between the two strains.

One potential therapeutic target gene in the brain that was differentially expressed in SHR compared to WKY neuronal cultures was adducin. Adducin is a ubiquitously expressed tetrameric cytoskeletal protein composed with either  $\alpha/\beta$  or  $\alpha/\gamma$  heterodimers, and is involved in intracellular protein trafficking,  $\text{Ca}^{2+}$  mobilization, and the phosphorylation state of certain kinases (208). Genetic variations in  $\alpha$ -adducin have been associated with primary human hypertension and the BP phenotype in Milan hypertensive rats (25,209–211). Polymorphisms of the  $\beta$  and  $\gamma$  subunits may contribute to BP variation, especially when associated with  $\alpha$ -adducin polymorphisms, consistent with the notion that biological activity of adducin is dependent on  $\alpha/\beta$  or  $\alpha/\gamma$  heterodimers (25,210,211).

A role for adducin in BP regulation has been further supported by the demonstration of higher BP in  $\beta$ -adducin deficient mice than in wild-type mice (212). We have identified a 22% decrease in  $\gamma$ -adducin (*Add3*) expression in SHR neurons compared with WKY neuronal cultures using gene expression profiling. Real-time RT-PCR confirmed the decrease in  $\gamma$ -adducin transcript, and Western blot analysis indicated a more dramatic decrease (approx 60%) in protein levels in SHR compared with WKY neurons in culture (206). This decrease in  $\gamma$ -adducin expression was maintained in the hypothalamus and brainstem of adult SHR and was also observed in mRen2 rats, suggesting that it is common to hypertensive models that exhibit an overactive brain RAS (206). Furthermore, Ang II treatment of either WKY or SHR neuronal cultures resulted in a decrease of  $\gamma$ -adducin transcript and protein levels, indicating a regulation of expression by Ang II. These observations demonstrate a decrease in  $\gamma$ -adducin expression in hypothalamic and medullary areas of hypertensive rats, and support the concept that an overactive brain RAS may be responsible for the decreased expression. We also evaluated the effect of  $\gamma$ -adducin inhibition on the firing rate of neuronal cultures. Using intracellular delivery of  $\gamma$ -adducin-specific antibodies to inhibit  $\gamma$ -adducin resulted in an increased neuronal firing rate, which was similar to that observed with Ang II. This effect was not additive, suggesting that a common mechanism resulted in the increased firing rate (206). We subsequently investigated two nongenetic models of hypertension, the chronic Ang II model and the desoxycorticosterone acetate model (213). In both models, the increase in BP was associated with a 70% decrease in hypothalamic  $\gamma$ -adducin; however, in contrast with the genetic models of hypertension, there was no change in brainstem  $\gamma$ -adducin. Neuronal cultures from the WKY strain of rats, when incubated with Ang II, resulted in a 60% decrease in the neuronal  $\gamma$ -adducin (213). Decreased  $\gamma$ -adducin may therefore contribute to augmented basal neuronal firing rate in cardiovascular-regulatory brain areas of hypertensive animals. Because central  $\gamma$ -adducin expression is decreased in SHR, we would predict that a gene therapy approach to overexpress this gene in the brain of the SHR may

decrease BP and reverse hypertension, whereas deleting or reducing its expression in WKY rats would induce hypertension.

Yu et al. (244) demonstrated an elevation in the expression of soluble epoxide hydrolase (sEH) from the kidney of the SHR when compared with the WKY. In addition, this group showed that blocking sEH lowered the BP in the SHR. Furthermore, Sinal et al. (214) reported that BP is reduced in a knockout mouse model for sEH, although this change in BP was observed only in male rats. However, when the kidneys from multiple strains of SHR and WKY rats were utilized in subsequent gene profiling studies no consistent changes in this gene were observed and there was no clear correlation between expression of the gene and hypertension (203,245). This led us to propose a novel hypothesis about the role of this gene in hypertension. Our hypothesis is that the expression of brain sEH may be key in the regulation of BP, and its altered expression or dysregulation could be better linked to hypertension. This hypothesis was tested and subsequently validated by demonstrating an upregulation of this enzyme in the hypothalamus and brain stem areas of the SHR by expression profiling analysis. In spite of the excellent physiological link between dysregulated expression of brain sEH and hypertension, direct gene transfer studies must be carried out to conclusively demonstrate the role of this enzyme in hypertension. It is from studies like these that gene profiling experiments that initially correlate changes in gene expression can move beyond to seek out mechanisms of functional significance.

### FUTURE GENE TARGETS OF THE RAS

The RAS is classically known as a hormonal system that is involved in salt and water regulation and BP control. The RAS is one example of a system in which dysregulated expression and hyperactivity have been associated with the development and maintenance of hypertension. Both the systemic (endocrine) and tissue (paracrine/autocrine) versions of the RAS contribute to hypertension (238,242). In the systemic RAS, a coordinated sequence of events involving various organs work together to generate circulating active hormone, Ang II. In this classical systemic endocrine system, angiotensinogen, which is produced in the liver, is converted to Ang I by the enzyme renin, which is secreted by the kidney. ACE is an enzyme that is found both circulating in the blood and membrane bound in numerous tissues including epithelial and endothelial cells (215,216), converts ANG I to ANG II. Various areas of the brain are also rich in ACE (217). Ang II produced as a result of the endocrine RAS is responsible for many short-term effects of Ang II, such as arterial vasoconstriction, aldosterone release, and sodium and water reabsorption by signaling through the AT<sub>1</sub>R (223,230,238). This classical understanding of the RAS led to the use of ACE inhibitors (ACEI) and Ang II receptor blockers (ARBs) as therapeutic agents in the treatment of hypertension. Because a hyperactive RAS is a key player in hypertension, and because 40–60% of hypertensive patients respond to ACE-inhibitors/AT<sub>1</sub>R-antagonists, one could hypothesize that genetic manipulation of this system to inhibit signaling within the RAS may, in principle, be an ideal method to attempt a genetic cure for this disease. We and others (62–67,73,74,84,91,93,99,101,102,104) have used an AS technique to downregulate transcription of the ACE enzyme and/or the AT<sub>1</sub>R to prevent the development of hypertension in both genetic and nongenetic models of experimental hypertension. However, this classical RAS is more complex, and new discoveries regarding angiotensin degradation fragments provide even more potential targets for gene therapy in hypertension.

Recently, the ACE2 enzyme has been characterized (218,219). This enzyme, which initially was found in the testis, kidney, and heart, has also been identified in a wide variety of tissues and is most likely localized, much like ACE (218–220). It shares 40% homology with ACE, but differs greatly in substrate specificity, and its activity is not altered by ACEI. ACE2 is one of several enzymes that catalyze the formation of degradation fragments angiotensin 1–9 (Ang 1–9) and angiotensin 1–7 (Ang 1–7) from both Ang I and Ang II, respectively (*see* Fig. 1). In mice lacking the *ACE2* gene, Allred et al. (227) observed a decrease in baseline BP and an enhanced pressor response during intravenous infusion of Ang II as compared with normal mice. BP was also decreased in animals that overexpressed *ACE2* (228). Therefore, the physiological effect of an imbalance of ACE2 over that of ACE could shift the RAS to increase vasodilator effects and reduce vasoconstrictor effects, thus resulting in a reduced BP (*see* Fig. 1). It is conceivable that the balance between ACE and ACE2 may be a pivotal mechanism in the regulation of BP and in the tissue pathophysiology (i.e., cardiac hypertrophy, renal disease) that has been associated with the tissue RAS. ACE2 also has the potential to be a better target than ACE because (1) it does not have the confounding interpretation effects that ACE does on bradykinin levels; (2) the gene has both a secreted form and a membrane-bound form that may allow us to differentiate BP effects from end-organ target pathologies; and (3) control of this unique enzyme can shift the emphasis of the entire RAS to one of vasodilation instead of vasoconstriction, and thus regulation of this enzyme may have significant therapeutic effects in the treatment of hypertension and other cardiovascular abnormalities.

Even though the actions of Ang II are best characterized, a role for other Ang peptides, such as Ang III, Ang IV, and Ang 1–7, as well as other Ang receptors, is rapidly emerging (223–225). Because ACE inhibitors and ARBs are effective in treating essential hypertension, it is conceivable that some of the actions of these drugs could be mediated through these nonclassical components of the RAS. Blocking the ACE enzyme, for example, causes bradykinin levels to increase, and this leads to a lowering of BP (226). Ang I levels would increase with ACE inhibition, and Ang II levels would increase with ARB treatment. In both cases, this could lead to an increase in Ang 1–7 via ACE2 and other endopeptidases. Ang 1–7 has been suggested to antagonize Ang II action directly at the AT<sub>1</sub>R as well as indirectly via other pathways (such as antagonizing ACE) and its concentrations are increased during ACE inhibition (221,222). Although Ang 1–7 is catabolized by ACE, it is also considered a competitive inhibitor of ACE (219). This new knowledge of the RAS suggests several potential genetic targets. Overexpression of ACE2, or increased production of Ang 1–7, are two future possible targets for gene therapy in the treatment of hypertension.

Most studies of the RAS have focused on the AT<sub>1</sub>R subtype. This subtype is one that is responsible for most of the known actions of Ang II, such as vasoconstriction, enhancement of noradrenergic neurotransmission, and release of hormones from the adrenal gland (229,230). There is an additional receptor subtype that can be activated by Ang II, the AT<sub>2</sub>R (229,230). This receptor, like the AT<sub>1</sub>R, is a G protein-coupled receptor with seven transmembrane regions (229–233). Although similar in size (363 amino acids for AT<sub>2</sub>R and 359 amino acids for AT<sub>1</sub>R), there is only 34% homology between the two receptor subtypes. The AT<sub>2</sub>R is more widely distributed in fetal tissues, and it has been suggested that this receptor is involved in developmental processes (229–233). Generally, the actions of AT<sub>2</sub>Rs oppose those of the AT<sub>1</sub>R (229–233). AT<sub>2</sub>R knockout models

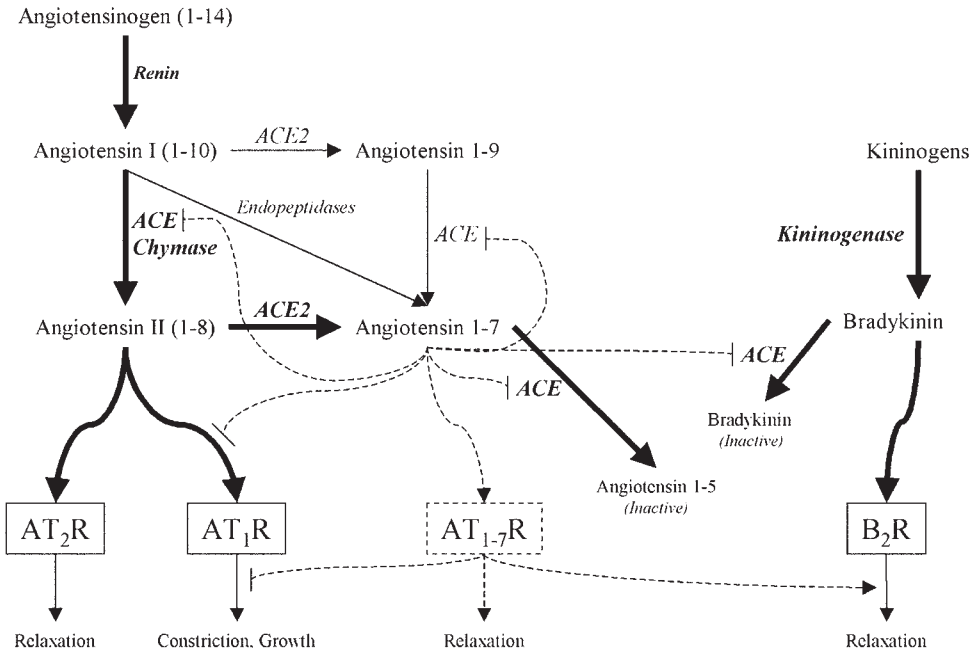


Fig. 1 The renin–angiotensin system. ACE, angiotensin-converting enzyme; AT<sub>1</sub>R, angiotensin type I receptors; AT<sub>2</sub>R, angiotensin type II receptors; B<sub>2</sub>R, bradykinin type II receptors.

exhibit increases in basal BP and/or show enhanced pressor responses to Ang II (234,235). Further, we have shown recently that overexpression of the AT<sub>2</sub>R inhibits cardiac hypertrophy in the SHR (59) and that underexpression of the AT<sub>2</sub>R can elevate BP in Sprague-Dawley animals (80). The recent finding that the AT<sub>2</sub>R can act in a constitutive manner (236) is also intriguing, and may suggest some clinical significance. Its use as a genetic target will continue to be examined.

In contrast to the classical systemic (endocrine) RAS, there is the local-tissue (paracrine/autocrine) RAS. Components of the classical, systemic RAS are found in numerous tissues (237–240). Ang II and its other active degradation fragments can be produced within given tissues, and thus have their physiological effects locally at that (or neighboring) tissues. This paracrine/autocrine system appears to play a major role in long-term cardiovascular regulation, especially in regulating cardiac hypertrophy and remodeling of the arteriole vasculature in other tissues (241–243). We have demonstrated that overexpression of the AT<sub>2</sub>R (59) or underexpression of the AT<sub>1</sub>R (62–64,91,93,99) is effective in preventing cardiac hypertrophy in genetic and nongenetic models of experimental hypertension. Targeting specific tissues with gene therapy approaches may be able to reverse and/or prevent the pathophysiological alterations that occur in hypertensive and other cardiovascular diseases. Figure 1 identifies most of the well-characterized components of the RAS, and thus identifies some current and future targets for gene therapy.

### SUMMARY AND PERSPECTIVES

Hypertension is a prevalent disease in which current intervention with pharmaceutical agents is effective in only about one-third of patients. Additionally, the current pharmacological approach aims to control BP, and will not lead to prevention or cure of the

disease. Gene therapy has the potential to prevent and/or cure hypertension, as clinical findings strongly support the notion that hypertension is genetically based. Many of these clinical findings involve components of the RAS, although there are many other candidate genes. Although there are numerous technologies available to use for discovery and examination of candidate genes, and for the delivery and manipulation of gene therapies, each has its own strengths and weaknesses. Current knowledge of the pathways that can lead to hypertension can suggest some of these potential genes, but this method will not reveal novel pathways or mechanisms to study and target. Microarray expression profiling studies can be used to identify a number of differentially expressed genes, but the challenge remains to determine the physiological relevance of these findings, and to identify which of these genes are linked to the development and/or maintenance of hypertension. The combination of gene expression profiling and the phenotypic characterization of *in vitro* and *in vivo* loss- or gain-of-function experiments has the potential to identify genes involved in the pathogenesis of hypertension, and thereby presents novel targets for therapy. Our primary research task then, still remains, to identify significant candidate genes.

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## Application of Viral Gene Transfer in Studies of Neurogenic Hypertension

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### SUMMARY

This chapter reviews the use of viral gene transfer to disentangle the complexities of neurogenic hypertension. Viral gene manipulation allows lasting and controllable genetic manipulation in selected areas of the brain in different species and strains, including experiments in the spontaneously hypertensive rat, an established model of hypertension. Recent evidence indicates that, in contrast to pharmacological tools that may act on any cellular target within a given area of the brain, viral vectors deliver transgene in a nonuniform manner, and its concentration in different types of cells may vary greatly. This occurs as a result of both transductional tropism of a viral vector system and the transcriptional activity of the promoter in different cellular types which are present in any brain nucleus. Properties of adenoviral and lentiviral vectors are compared and contrasted. Application of viral vectors for overexpression of biologically active molecules, expression of dominant negative proteins, pathway tracking, and other experiments to study central mechanisms of cardiovascular control are discussed. In summary, virally mediated gene delivery to the brain is a powerful research tool that can be used to address a wide range of questions related to mechanisms of human essential hypertension.

**Key Words:** Hypertension; viral vectors; adenovirus; gene expression; gene targeting.



## INTRODUCTION

In spite of recent advances in the treatment of hypertension, many patients fail to respond to standard therapies targeted mainly at regulating blood volume, electrolyte balance, and the peripheral renin-angiotensin system. In a recent review, S. J. Mann writes: "One such form of hypertension is the often overlooked entity of neurogenic hypertension. These implications *underscore the need for further clinical and basic research attention concerning neurogenically mediated hypertension*" (1). The purpose of this chapter is to renew attention to this overlooked entity by considering novel molecular strategies to disentangle the complexities of neurogenic hypertension.

The lack of understanding of the mechanisms of neurogenic hypertension to which S. J. Mann alludes justifies interest in new methods of analysis of this disease, which would allow us to study these mechanisms with a new level of precision. Many such methods have evolved during the last decade or so, and currently converge under the umbrella of physiological genomics. Availability of fully sequenced genomes and the vast numbers of readily accessible clones greatly facilitates the use of genetic manipulations in order to reveal the roles of individual genes and their families under normal, as well as pathological, conditions.

We have chosen to employ viral vectors to manipulate genes within the central nervous system structures regulating blood pressure. Virally mediated gene delivery to the brain is a powerful research tool, which can be used to address a wide range of questions including those concerning central cardiovascular control mechanisms. So, what are the advantages of the viral approach?

### *Why Viral Vectors?*

First, viral vectors allow evaluation of the long-term outcomes of various experimental manipulations on blood pressure control. It is not a secret that many conclusions about central mechanisms of blood pressure control have originated from acute experiments in anesthetized animals. Meanwhile, human hypertension is a chronic condition and, thus, it becomes imperative to also look at long-term manipulations within specific brain structures for a more complete understanding of this condition. In addition, acute experimentation usually occurs under anesthesia which has very profound effects on nearly every transmitter system relevant to blood pressure control (reviewed in ref. 2). Nowadays, chronic recording of arterial blood pressure using radiotelemetry in rats or mice has negated these problems. However, one problem that remains is the chronic central delivery of pharmacological agents directly into specific brain nuclei. Here, viral gene manipulation is of great advantage because, following a single microinjection, it enables chronic alteration of brain signalling in a chosen structure that can last for many days or even weeks (3–8). Moreover, in experiments of this kind it is possible to obtain an intraexperimental control (e.g., monitor blood pressure before and after the genetic manipulation in the same animal). This is important, because it greatly facilitates interpretation of the results (*see* section titled "Application of Somatic Gene Transfer in Studies of Central Mechanism of Blood Pressure"). In addition, pharmacological approaches are, in many cases, impossible because of the lack of suitable drugs, especially for recently discovered targets and for those awaiting discovery.

Second, viral gene manipulation is a flexible approach, because the same vectors can be used in many species. There is little doubt, for a variety of reasons, that the rat will remain the species of choice for neuroscience studies including those concerned with

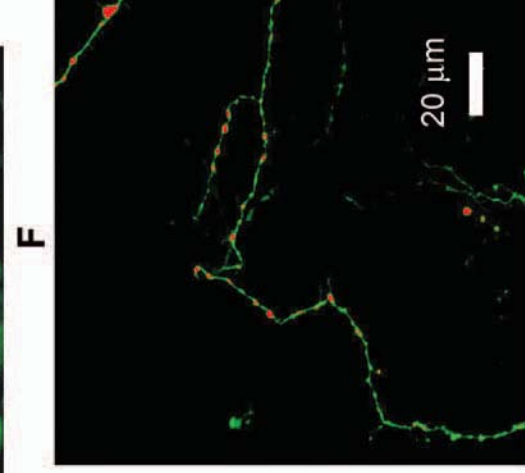
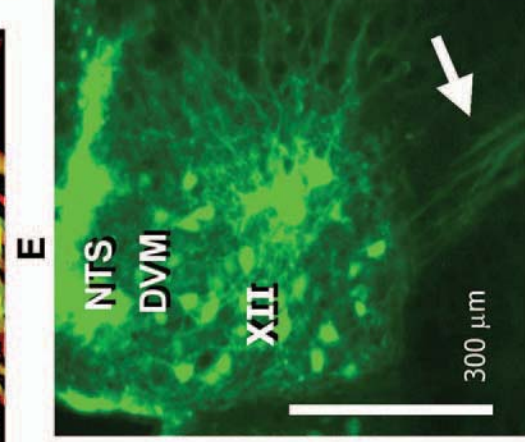
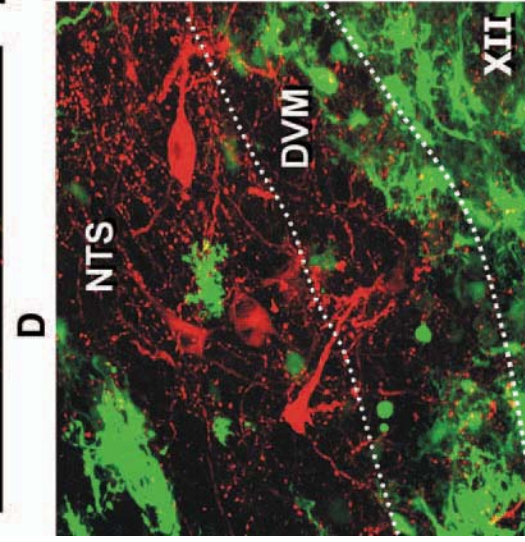
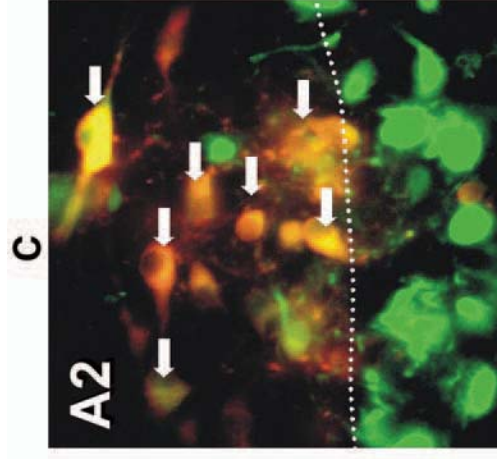
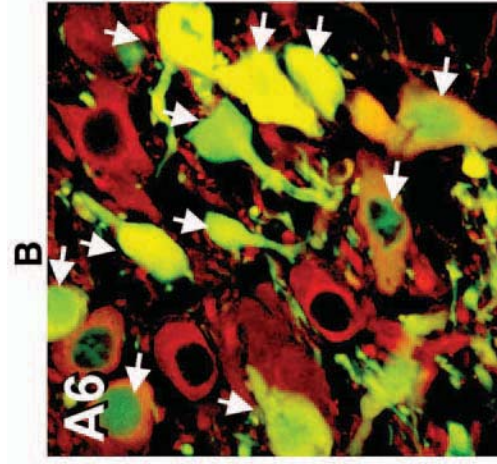
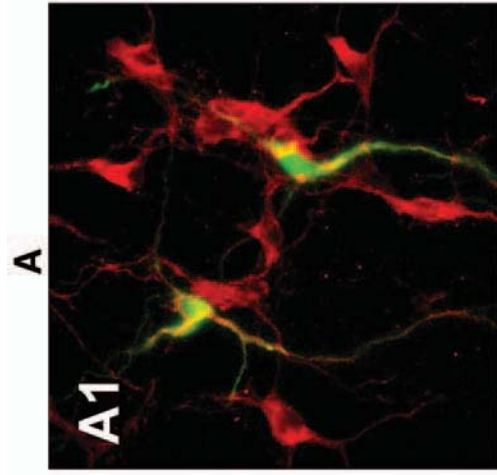
hypertension. In the context of this chapter, it is a great advantage that this approach can be applied to the spontaneously hypertensive rat (SHR) and the outcome compared directly with its normotensive progenitor strain, the Wistar–Kyoto rat (WKY), or any other control strain (3,9). Whereas germline transgenesis in the rat is extremely technically challenging and time-consuming, viral gene delivery does allow effective manipulation of central signaling pathways (10). Although the SHR is not an ideal model of essential hypertension in humans, it has nevertheless served for many years as a valuable model of this disease and, with its normotensive progenitor control WKY, remains an important resource. Moreover, in addition to SHR, there are many other models of hypertension in the rat.

Third, viral gene transfer enables both site-specific and, in some cases, cell-type-specific genetic manipulation. Viral vectors can be delivered stereotaxically into any area of the brain and, if needed, retrograde transduction performed with some types of vectors (11–13) (Fig. 1). However, all brain nuclei are heterogeneous in terms of their histochemistry and connectivity. Many nuclei harbor excitatory glutamatergic and inhibitory  $\gamma$ -aminobutyric acid (GABA)-ergic neurons, and changes in both of these transmitter systems have been implemented in pathological hypertension (14–18). Additionally, catecholaminergic cell groups (e.g., A2 in the nucleus tractus solitarius [NTS], A6 in the Locus Coeruleus, C1 in the rostral ventro-lateral medulla) have all been implicated in pathological hypertension (19–23). Moreover, each group of neurones is embedded in a network of glia and blood vessels containing endothelial and vascular smooth muscle cells. Thus, it now becomes necessary to selectively target a cell type with a specific phenotype within a heterogeneous population using modified viral delivery vectors with cell-specific promoters and analyze their exact roles in pathological hypertension.

Finally, we should not forget that a large volume of information about the biochemical changes in central signaling mechanisms in hypertension has been obtained in highly reductionistic models, such as cell lines. For example, the PC12 line has been derived from pheochromocytoma and used as a model of central catecholaminergic neurones. Cell lines such as these are convenient in particular because DNA can be easily introduced using well-established methods, such as calcium phosphate transfection or liposome-based carriers. However, central neurones are very different from these simple models and to make a definitive conclusion about the function of brain circuits, genetic manipulation must also be made *in vivo*. Central neurones are generally very resistant to chemical methods of gene delivery, and here viruses again offer a powerful alternative. For example, if a hypothesis links hypertension to a change in a signaling cascade in catecholaminergic neurones of a particular cell group within the brainstem, viral vectors make it possible to perform a variety of experiments specifically on this cell type.

### ***Nonuniform Transductional and Transcriptional Efficacy of Viral Vectors***

We would like to draw the reader's attention to one very important feature of viral gene transfer experiments: the nonuniformity of transgene expression. Pharmacological analytical tools injected into the central nervous system affect all cells that have the appropriate binding site or receptor. In contrast, virally delivered transgenes may or may not be effective in some cells depending on the efficiency of the virus at both entering the cell and inducing expression of the transgene once it has reached the nucleus. The ability of the virus to enter the cell and the subsequent transcriptional activity of its expression cassette are two critical rate-limiting steps. Indeed, this gave rise to the concept of targeted



viral gene delivery, which can occur at either a transductional (i.e., at the stage of viral entry) or transcriptional level. Detailed analysis of viral targeting is outside of the scope of this chapter, and more information can be found in recent reviews (24–27). It is essential, however, to realize that as a result of the natural tropism of viral capsids (and/or the protein coat of enveloped viruses), and the striking variability of promoter activity in different neuronal populations (reviewed in more detail in ref. 27), the transgene probably is *never* expressed at the same level in all cells in the brain area in which the viral vector was injected. Indeed, a “false negative outcome” can occur if the transgene is expressed at very low, physiologically insufficient levels. On the other hand, expression of a biologically active molecule in cells that normally do not express this molecule may alter their function in an unpredictable manner. These considerations are important for interpretation of viral gene transfer experiments and have led us to perform a more thorough characterisation of the expression profile of a number of viral vectors in cardiovascular areas of both the brainstem and diencephalon (27,28).

In this chapter we will give an overview of currently available viral gene delivery systems and illustrate how they can be used to advance our understanding of the central mechanisms of hypertension.

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**Fig. 1.** (A) Retrogradely transfected noradrenergic neurones in the A1 brainstem area. Adenoviral vector (AVV) PRSx8-enhanced green fluorescent protein (EGFP) ( $1\ \mu\text{L}$  of  $2.2 \times 10^8$  PFU) was injected in the paraventricular area of the hypothalamus. Five days later, sections were prepared and immunostained for dopamine  $\beta$ -hydroxylase (DBH; red fluorescence of the CY3 conjugated secondary antibody). This image is an overlay of the EGFP and CY3 channels obtained separately using spectral confocal microscope. Note that both EGFP-expressing neurones are also DBH-positive, and therefore appear yellow. (Reproduced from Kasparov et al. [27], with permission). (B,C) AVV PRSx8-EGFP was injected into either Locus Coeruleus (A6) or dorsal vagal complex (including the A2 group). Six days later, the tissue was fixed and processed for DBH. The images are overlays of EGFP (green) and DBH (red) channels of confocal images. Note that in the A6, all transduced neurones appear yellow (arrows) owing to co-localization with the DBH stain. In contrast, in the dorsal vagal complex, whereas the EGFP-expressing cells in nucleus tractus solitarius (NTS) were DBH-positive A2 cells (yellow color, thick arrows), the underlying neurones of the dorsal vagal motor nucleus (below the dotted line) also expressed high levels of EGFP (green color, DBH-negative). Data contributed by D. Lonergan (University of Bristol). (D,E) AVV HCMV-EGFP expression profile in the dorsal vagal complex. Two injections of  $0.5\ \mu\text{L}$  of  $2.7 \times 10^8$  PFU of the vector were made at 300 and 400  $\mu\text{m}$  from the dorsal surface of the medulla. The tissue was then processed for DBH. The NTS is devoid of EGFP-expressing neurones; no EGFP-expressing cells in NTS area were DBH-positive. Some glial cells can be seen. In contrast, there are numerous EGFP-expressing cells (both glia and neurones) in the deeper cholinergic motor nuclei. In particular, many of the neurones of the motor nucleus of the hypoglossal nerve expressed EGFP. These cells could be easily identified on low-power images (right panel) by their long axons projecting toward the ventral edge of the medulla. (F) AVV PRSx8-EGFP was used to visualize catecholaminergic neurones and their beaded axons in organotypic slice cultures of the rat brainstem. Vectors were applied to plating media and fluorescent neurones visualized after more than 3 d using conventional and confocal fluorescent microscopy. (Further details in ref. 27.)

## VIRAL VEHICLES FOR GENE DELIVERY INTO CENTRAL NEURONES

Vectors for gene delivery into the brain have been derived from a number of viral genomes, such as adenoviruses, adeno-associated viruses (AAVs), retroviruses and related lentiviruses, herpes simplex virus, and some others. So far, studies of central mechanisms of cardiovascular control have mainly employed adenoviral vectors (AVV) and retroviral vectors, including lentiviral vectors (LVV) (3,5,10–12,29–33). These viruses can infect various cell types present in the brain (i.e., neurones, glia, endothelial, and vascular muscle cells) and result in long-lasting gene expression without noticeable adverse effects. Vectors derived from the AAV have been successfully used for gene delivery to peripheral targets (34,35), but they are also well-equipped for gene delivery into central neurones (36). Finally, a replicating pseudorabies virus has been used to retrogradely transfect vagal cardioinhibitory neurones (13).

### *General Properties of AVV and LVV*

AVV and LVV are very different in respect to their genomic organization, structure, and life cycle. Whereas AVV vectors are descendants of the widespread pathogens associated with mild human infections of the respiratory tract and eyes, LVV vectors have been derived from either a deadly human immunodeficiency virus (HIV) or a feline immunodeficiency virus (FIV). From the practical point of view, the most important features of any viral gene delivery system include its safety in use (i.e., nonvirulent), cellular tropism in the brain, transgene carrying capacity, rapidity, longevity and stability of gene expression, the degree of an immune response induced, the ability for retrograde transduction, and the speed and cost of production.

In contrast to AVV, LVV are able to stably insert the transgene into the host genome, whereas the transgenes delivered by AVV remain episomal. Thus, AVV-delivered transgenes may be relatively rapidly eliminated (2–3 wk), especially in dividing cells, such as endothelial cells, whereas LVV induce expression for months. In central neurones, this difference might be less evident because the longevity of expression seems to be largely dependent on the promoter used. Some AVV constructs have been reported to drive expression for at least 9 mo (37). In terms of capacity, currently the majority of laboratories use AVV with deletions in the E1 region (with additional deletions in the E2 and/or E3 and E4 regions). With these deletions, which render the virus replication deficient, AVV can accommodate approx 7 kb of a transgene. This is enough for one or even two expression cassettes. Various versions of AVV have been made, some of which express two different proteins from two separate promoters (38). In addition, high-capacity AVV lacking most of their genome have been generated (39), but production of these vectors remains too complicated for them to replace the currently used E1-E2/E3 deleted AVV. In contrast, HIV-derived self-inactivating LVV, such as those developed by Iwakuma et al. (40) and further modified by Coleman et al. (41), have most of the viral genome deleted and, in principle, can accommodate approx 11–12 kb. Size limitations within the viral backbone are important, particularly when large transgenes are inserted or when cell-specific expression is desirable. To achieve cell specificity in transgenic animals, researchers commonly use long promoter sequences (several kb) upstream of a particular gene to drive the expression (42) or even bacterial artificial chromosomes (tens of kb; [43]). Clearly, viral vectors have restrictions imposed by the size of viral particles, but it proves possible to target expression to at least some subsets of cells relevant to

central cardiovascular control (*see* section titled “Future Potential of Viral Gene Transfer,” and Fig. 4). Parenthetically, in contrast to AVV and LVV, AAV have even smaller capacity. Even the most advanced versions of these vectors accommodate inserts no larger than approx 4 kb, which limits their use for expression of large cassettes (44).

#### TRANSDUCTIONAL TROPISM OF THE AVV AND LVV

AVV particles are very different from LVV in that they are nonenveloped and their ability to invade cells is dependent entirely on the proteins present in their fibers and capsid. It has been demonstrated that the serotype affects the neuronal tropism of the AVV, and apparently fibers of the Ad17 serotype might be best suitable for targeting neurones (45). Nevertheless, most of the AVV vectors currently used have been derived from serotype 5. Adenovirus fiber protein interacts with a range of protein receptors present on the membranes of many cells, such as the Major Histocompatibility Complex class I molecule and the “Coxsackie–adenovirus” receptor (CAR). Modifications of the AVV fiber protein may change its tropism and alter its ability to infect certain cell types (for review, *see* ref. 26). Recently, using this approach, Omori et al. (46) have redirected AVV to microglia which lack CAR. This was achieved by incorporating the Arg-Gly-Asp motif containing peptide into the HI loop of the fiber knob. This motif enables interaction with  $\alpha$ V integrins, ubiquitously expressed on the surface of mammalian cells. Similarly, Baker and his colleagues were able to modify the HI loop to detarget AVV from CAR and, instead, direct it to endothelial cells (47,48). Thus, modifications of adenoviral capsid can lead to the development of vectors with an even better ability to preferentially target different cell types present in brain, such as glia, endothelium, or, perhaps, neuronal subsets. Thus, alteration of vector tropism may be achieved by modification of the capsid proteins.

#### AXONAL TRANSPORT AND TARGETING REMOTE SITES IN THE BRAIN

The ability of a viral vector to transduce remote neurones via retrograde transport may have major implications for its use in experimental neuroscience. This feature is heavily dependent on the properties of the viral particle, but could also be influenced by the phenotype of the targeted neurones. There has been some controversy regarding the ability of the conventional AVV (serotype 5) to transduce neurones retrogradely, but this issue has been recently resolved by a number of studies. For example, through the use of AVV with the Rous sarcoma virus long terminal repeat as the promoter, retrograde transduction was obtained after microinjections into the caudate putamen (49). Transfected cells (predominantly dopaminergic) were found in substantia nigra compacta that was 2 mm from the site of injection, ruling out viral diffusion as the method of transfection. Nashimura et al. (50) achieved retrograde transfection through intrahippocampal AVV injections using a modified chicken  $\beta$ -actin promoter (51) rather than human cytomegalovirus (HCMV). Neurones retrogradely transduced in these experiments were located in the cerebral cortex and were likely to be glutamatergic. Irnaten et al. (12) used AVV with an HCMV-driven construct to retrogradely transduce, from the pericardial sac, cholinergic neurones in nucleus ambiguus located in the ventrolateral medulla. Sinnayah (11) found retrogradely transfected neurones in the subfornical organ after injection of AVV with the HCMV promoter into the supraoptical nucleus (the exact transmitter phenotype of these cells is not known). Collectively, these data indicate that AVV *are* able to retrogradely invade various types of neurones that have different neurochemical profiles. Finally, we have also observed retrograde transduction of noradren-

ergic and adrenergic cells using AVV with the PRSx8 promoter, which is highly active in these cell types. For example, after injection of AVV PRSx8-enhanced green fluorescent protein (EGFP), into the paraventricular area of hypothalamus in the rat, noradrenergic neurones expressing EGFP were found in cardiovascular regions of the NTS and in the A1 area of the ventrolateral medulla (Fig. 1A). Moreover, injection of the same construct into the dorsal horn of the spinal cord resulted in EGFP-expressing noradrenergic neurones in locus coeruleus and some ventral brainstem noradrenergic groups. Therefore, we believe that transgene expression in remote sites after AVV microinjections may occur within the central nervous system, provided that the promoter used is active in these projection neurones.

In contrast with AVV, LVV have an additional protein coat or envelope wrapped around the capsid. The coat is associated with the capsid but consists of a separate protein encoded by separate genes. Because this coat is involved in the binding of the viral particle to the cellular membrane, modifications of the coat can alter the transductional properties of the vector, just as we described above for AVV. Most vectors used currently, irrespectively of the virus they have been derived from (i.e., HIV or FIV), are pseudotyped in that they employ a foreign protein coat, such as the vesicular stomatitis virus glycoprotein G (VSVG) (11,40,41). The VSVG coat enables LVV to deliver transgenes to a wide range of cells including neurones and glia (41). Pseudotyping with other protein coats generates new properties. The VSVG-pseudotyped vectors seem to have negligible ability for retrograde transduction. In contrast with an AVV, for example, VSVG-pseudotyped FIV did not retrogradely transduce neurones in the subfornical organ when injected into the supraoptical nucleus (11). This feature of VSVG-pseudotyped LVV may be important for functional studies when gene manipulation needs to be restricted to the injected brain area. Additionally, when an elongation factor 1 $\alpha$ (EF1 $\alpha$ )-EGFP LVV with the VSVG envelope was injected into the hypothalamus of the rat, no GFP-expressing neurones in remote areas, such as the brainstem, could be found (Kasparov, Paton, Huentelman, and Raizada, unpublished observation). Other coat proteins, such as the rabies-G envelope protein, however, alter this property permitting retrograde axonal transfection as demonstrated with the equine infectious anemia virus-bearing HCMV- $\beta$ Gal expression cassette (52). A good example of how the type of coat protein may change the cellular tropism of the LVV in the brain is that of the Ross River virus glycoproteins changing targeting by the FIV from neurones (characteristic of VSVG coat) to glia (53).

#### **AAV VECTORS FOR GENE DELIVERY IN THE BRAIN**

AAV used currently have been derived from the serotype 2 AAV. These viruses have a small, nonenveloped capsid and are efficient in transducing central neurones (54–57). Their tropism to other potentially interesting cell types, such as endothelial vascular cells, is lower than that of the AVV but can be increased by a capsid modification (57). Recently, it has been demonstrated that as with the AVV, AAV can also transduce neurones retrogradely: for example, expression occurred in the entorhinal cortex after injection into the hippocampus (58). AAV lack almost their entire native genome and therefore do not cause expression of their capsid proteins in transduced cells. Because capsid proteins can trigger an immune response, these vectors are advantaged by this property, making them particularly suitable for systemic use in tissues outside of the brain–blood barrier. For some time, it had been thought that AAV integrate their transgenes into the host chromo-

some, similar to the wild-type AAV, but this does not seem to apply to vectors derived from it. Thus, AAV-delivered transgenes remain largely episomal. Nevertheless, the expression following AAV transduction usually lasts for months which, together with an absence of any immune response, makes these vectors one of the favorite prototypes for gene therapy research. For further information on AAV vectors, see recently published reviews (27,56,59,60).

## APPLICATIONS OF SOMATIC GENE TRANSFER IN STUDIES OF CENTRAL MECHANISMS OF BLOOD PRESSURE CONTROL

In the following, we describe the types of experiments that can be performed using viral vectors that are capable of efficient gene delivery into central structures regulating cardiovascular homeostasis.

### *Overexpression Experiments*

A common experiment is to overexpress a certain protein in a brain structure of interest in order to increase the activity of this protein and monitor the outcome chronically. This approach has been used in a number of studies by Hirooka and colleagues (5,61–65). For example, in (1) an AVV with HCMV promoter was used to overexpress the endothelial isoform of nitric oxide synthase (eNOS) in the rostral ventrolateral medulla (RVLM), a major region providing descending sympato-excitatory drive. The experiments were carried out in two strains of rats—the stroke-prone SHR and its progenitor strain, the WKY rat. Arterial pressure was chronically monitored using radiotelemetry. Seven days after gene transfer, mean arterial pressure and heart rate decreased in both groups but these falls were significantly larger in the SHR. In addition, baroreceptor reflex input–output function was increased in both rat groups. A control for the nonspecific effects of the viral transduction was made using an AVV expressing  $\beta$ -galactosidase, which caused no change in the parameters measured. The authors argued that an increase in nitric oxide (NO) production in RVLM improves the impaired baroreceptor reflex control in SHR. In a previous study from the same group (65), eNOS was overexpressed in the NTS. The increased production of NO was demonstrated using microdialysis. In eNOS-transfected animals, blood pressure and heart rate were significantly decreased 5–10 d after gene transfer.

In these studies, the cellular targets to which the transgene was delivered was not established: it is not known whether the bulk of the eNOS-expressing cells were neurones, glia, or vascular cells (smooth muscle or endothelium). NO is thought to freely diffuse through cellular membranes and may reach targets in cells located some distance away from the source of release. But how far it travels under physiological conditions is not entirely clear (66). In the experiments described above, eNOS activity will appear in the cells determined by both the transductional tropism of the AVV and the transcriptional activity of HCMV promoter. This is very likely to be a different subset of cells from the ones producing eNOS endogenously. In the NTS, AVV with the HCMV promoter largely targets glial cells, and transgene expression in local endothelial cells has also been documented (*see ref. 27*). Because NO can easily cross cellular membranes, its overproduction in these experiments means that it could still reach its receptor (soluble guanylate cyclase) located in cells adjacent to the transfected cells. However, if the overexpressed molecule had to act on the target within the same cell, any mismatch between the expression profile of the viral vector and the distribution of the target molecule could lead to a false negative



or otherwise unpredictable outcome. Moreover, the action of high local concentrations of NO on the targets *within* the cells in which the AVV causes eNOS overexpression might result in nonphysiological responses.

In summary, overexpression of eNOS will produce large quantities of NO comparable to a chronic infusion of an NO donor into the structure. This provides a good method of chronic delivery of a substance without the need of repeated injections into a single brain locus, which is technically unachievable in many brain structures. However, both overexpression of eNOS and delivery of a pharmacological agent are unlikely to accurately mimic the physiological scenario in terms of providing a site- or cell-specific source of NO at a physiologically relevant concentration.

AVV with the HCMV promoter was also used to overexpress mitochondrial or cytoplasmatic superoxide dismutase isoforms to demonstrate involvement of reactive oxygen species (ROS) in the pressor, and dipsogenic responses induced by intracerebroventricular (icv) administration of angiotensin II (Ang II) in mice (8). ROS have been implicated in the pathogenesis of neurodegenerative disease but, in addition, are currently thought to act as second messengers in some signaling systems. In the Zimmerman et al. study (8), AVV were administered in the lateral ventricle of the mouse brain and the outcome evaluated 3 d later. Arterial pressure and heart rate were monitored before and after intraventricular administration of Ang II. Overexpression of both mitochondrial and cytoplasmatic isoforms strongly antagonized responses to icv injections of Ang II. The dipsogenic action of Ang II was also significantly antagonized. These studies have established a role for ROS in an AT<sub>1</sub> receptor-mediated signaling pathway regulating the cardiovascular system. The transgene in these studies was found predominantly in the brain tissue surrounding the ventricles and the subfornical organ, a circumventricular organ with a high density of AT<sub>1</sub> receptors known to be involved in blood-to-brain communication. Thus, AT<sub>1</sub> receptors in the subfornical organ may trigger hypertension via signaling cascade involving ROS. More recently, the same group has found that overexpression of Cu/Zn superoxide dismutase in subfornical organ and organum vasculosum of the lamina terminalis (this was achieved using icv injection of AVV) reduced sympathetic tone in mice with cardiac failure triggered by myocardial infarction (67). Interestingly, ROS scavenging did not affect the sympathetic tone in control animals without cardiac failure, suggesting that ROS signaling becomes activated only under these pathological conditions. The authors conclude that in heart failure, ROS within the circumventricular organs may mediate central sympathoactivation by circulating molecules such as Ang II and aldosterone (67).

We used a similar strategy to address the role for ROS in the AT<sub>1</sub>-receptor mediated signaling in the NTS, except that we overexpressed catalase instead of superoxide dismutase. We also used an AVV with HCMV promoter, but in our case this approach failed to prevent the depressant action of Ang II on baroreceptor reflex-mediated bradycardia in spite of the significantly elevated catalase activity in the area of transfection (10). Clearly, this reflects differences in the signalling mechanisms employed by the same receptor in the two different brain areas. However, the methodology of viral gene transfer leaves room for alternative interpretations that have to be considered. First, is it possible that in the NTS, AVV with the HCMV promoter did not lead to sufficient catalase expression in the cells that mediate the AT<sub>1</sub> receptor-mediated baroreceptor reflex attenuation? The precise nature of these cells is still unclear, but our current evidence suggests that they could be the eNOS-bearing endothelial cells. From our

experiments, in which eNOS dominant negative protein, expressed using an AVV with HCMV promoter, was able to antagonize AT<sub>1</sub> receptor-mediated effects in NTS (*see* Figs. 2 and 3), we reckon that this is unlikely to be the case. Second, it is not impossible that the catalase expressed was inefficient in inactivating the ROS generated by AT<sub>1</sub> receptor activation in the NTS. This argument is more difficult to dismiss, but we could argue that the lack of the effect of the catalase overexpression was consistent with the inability of a number of pharmacological blockers to antagonize various steps in the cascade signaling pathway downstream of ROS generation (10).

In summary, these studies illustrate some of the potential experimental applications of virally mediated transgenesis for overexpression of a protein in different central nervous structures to evoke chronic alterations in cardiovascular function.

### ***Expression of Antisense Oligonucleotides and siRNA-Mediated Gene Suppression***

Viral vectors have also been used in “loss-of-function” experiments in order to chronically decrease expression of a certain gene. There is more than one way to achieve this. Lu and Raizada (68) successfully used a retroviral vector to express an antisense sequence to the 5' end of the AT<sub>1B</sub> receptor gene. The vectors were applied to primary cultures from brainstem to down-regulate AT<sub>1</sub> receptor expression. This experiment resulted in downregulation of all measured responses to Ang II, including c-fos and noradrenaline transporter expression.

Although the antisense approach has been used successfully by some groups (35,68,69), the more recent reports about small interfering RNAs (siRNA) being able to selectively target specific mRNA species into the degradation pathway have evoked huge interest from the scientific community (70,71). Most published studies to date have employed synthetic double-stranded RNA oligonucleotides (72–74). However, it is also possible to use viral vectors to express siRNA to the same effect (75–78). These vectors are designed in such a way that transcription results in generation of hairpin-like RNA, which acts similarly to the conventional siRNA. Viral vectors for expression of siRNA have been made on the basis of polymerase II promoters, such as HCMV (78), and polymerase III promoters such as U6 (75) and H1 (79). Polymerase III promoters are currently thought to be optimal for siRNA hairpin expression because they provide a high rate of transcription of small RNA molecules in a wide range of cells. Moreover, the mechanisms of the initiation and termination of transcription by polymerase III allow production of precise lengths of transcripts that do not undergo mRNA-specific posttranscriptional modifications, which interfere with siRNA function. Some systems of that kind are available commercially. Viral vectors with polymerase III-derived inducible siRNA expression systems have also been developed (79). At this point, this technology has not been applied in studies of central cardiovascular control, but this is just a matter of time.

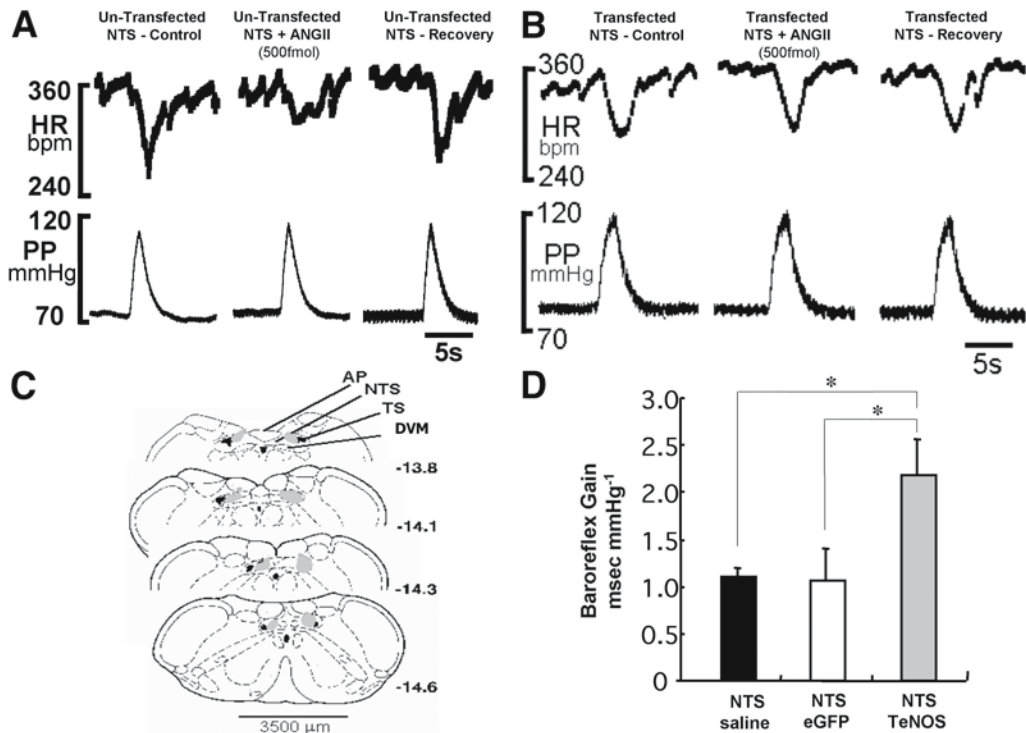
### ***Expression of Dominant-Negative Proteins***

It is possible to suppress the function of certain proteins by expression of dominant negative proteins. This principle usually employs protein–protein interaction, for example, between the dominant negative protein and a catalytic subunit of an enzyme. We were interested in the role of NO in baroreceptor reflex control at the level of the NTS. A number of pharmacological tools were used (32) to demonstrate that various blockers

of NO production prevented a well-documented inhibitory action of Ang II on this reflex in the NTS (80,81). Viral gene transfer of a dominant negative form of eNOS was then employed for two reasons. First, we believed that it would be more selective for the “endothelial” NOS isoform (as opposed to the “neuronal” or nNOS isoform) than the available pharmacological antagonists. Second, it was important to evaluate the role of NO in NTS chronically, and long-term pharmacological blockade of eNOS in NTS is technically not achievable. To suppress endogenous eNOS in the NTS, an AVV was used to express a truncated form of eNOS (TeNOS) under the control of the HCMV promoter (82). Although TeNOS lacks catalytic activity, it is correctly localized to the membrane. TeNOS acts as a dominant negative inhibitor of wild-type eNOS activity through heterodimerization with the native protein (83). We demonstrated that in animals that had received three bilateral injections in the NTS of the TeNOS AVV 5–6 d prior to the test, Ang II, which normally strongly suppresses baroreceptor reflex when administered into the NTS, was ineffective (Fig. 2) (32). Similarly, the NO precursor L-arginine, which also inhibited the baroreceptor reflex when injected into the NTS of naïve rats, failed to modulate the baroreceptor reflex in TeNOS-transfected animals. There appeared to be no evidence of any aberrant effects of the AVV because: (1) the baseline baroreceptor reflex sensitivity was not altered by TeNOS expression; and, (2) an EGFP-expressing AVV did not cause any detectable alterations in baroreceptor reflex function and multiple other physiological variables measured.

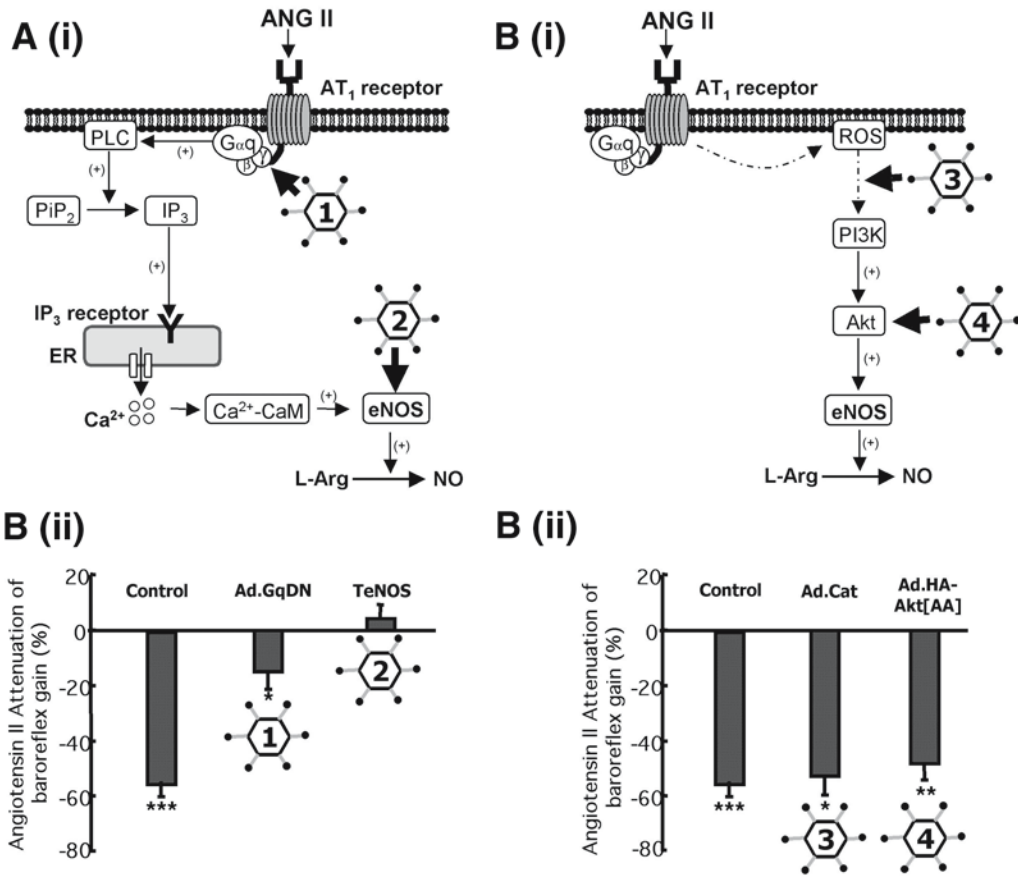
More recently, the same construct was used chronically in normotensive conscious freely-moving rats instrumented with radio transmitters to record their blood pressure and heart rate. This approach gives a continuous definitive measure of arterial pressure. After the implantation of transmitters, the basal level of blood pressure, heart rate, and spontaneous baroreceptor reflex gain were determined by a time-series method. Subsequently, the animals were then reanesthetized and the AVV-TeNOS microinjected into the NTS. TeNOS expression evoked a gradual increase in baroreceptor reflex gain between d 7 and 21 after gene transfer, peaking at d 21 ( $1.68 \pm 0.20$  ms/mmHg). This value was significantly higher compared to that before gene transfer ( $1.13 \pm 0.09$  ms/mmHg). It was also significantly elevated compared with two groups of control animals: those that received an NTS microinjection of an AVV expressing EGFP, which acted as a viral control, and those that received a microinjection of saline. In addition, heart rate decreased 14–21 d after *TeNOS* gene transfer. However, blood pressure was not affected. These results indicate that eNOS is constitutively active within the NTS, and acts to regulate baroreceptor reflex gain and heart rate but not resting blood pressure in normotensive WKY rats (3). In addition, these data suggest that baroreflex gain (at least the cardiac component) is controlled independently from arterial pressure by the NTS. This prompted the question as to whether eNOS activity in NTS of the SHR was important for the known depressed baroreceptor reflex gain in this animal model. Our preliminary data indicate that when TeNOS was expressed in 10- to 11-wk-old SHR, baroreceptor reflex sensitivity increased, whereas systolic blood pressure decreased (84). These results suggest that in this model of pathological hypertension, endogenous eNOS activity in the NTS is elevated compared with normotensive rats and plays a major role in determining levels of *both* arterial pressure and baroreceptor reflex gain. For reasons which will become apparent later, it is worth mentioning that in all of these vectors, transgene expression was driven by the HCMV promoter.

We have also used AVV expressing dominant negative proteins to dissect out the intracellular signaling pathway utilized by Ang II to suppress the baroreceptor reflex



**Fig. 2.** Examples of the usefulness of adenoviral vector (AVV)-mediated expression of a dominant negative protein in the nucleus tractus solitarii (NTS) for cardiovascular control. Angiotensin II (Ang II) injections into the NTS attenuate the reflex bradycardia to baroreceptor stimulation (**A**). This was shown to be dependent on endothelial nitric oxide synthase (eNOS) because adenoviral transfection with a dominant negative “TeNOS” (a truncated form of eNOS) prevented the effect of Ang II (**B**). Transfection sites (indicated by grey shading) were localized within the NTS as revealed by expression of enhanced green fluorescent protein (EGFP) using a second adenovirus (**C**). Chronic expression of TeNOS in rats fitted with telemetry devices to record arterial pressure revealed a significant elevation in baroreceptor reflex sensitivity in normotensive rats (**D**). Note that neither saline injections into NTS or expression of EGFP in this nucleus altered baroreceptor reflex gain. AP, area postrema; DVM, dorsal vagal motor nucleus; HR, heart rate in beats per min (bpm); PP, perfusion pressure; TS, solitary tract. (Data taken from refs. 3 and 32.)

within the NTS (10). In contrast, HCMV-EGFP-injected animals were no different from naïve rats in terms of Ang II action on the baroreceptor reflex in NTS. However, expression of a dominant negative Gq protein driven by the human elongation factor (EF1 $\alpha$ ) promoter in NTS strongly antagonized the depressant action of Ang II on the baroreceptor reflex (Fig. 3). This was an important finding because Gq is one of the established intracellular links between G protein-coupled receptors, such as the AT<sub>1</sub> receptor, and intracellular Ca<sup>2+</sup> stores. These data were consolidated using conventional pharmacological approaches to block intracellular signalling pathways (10). In the same study, some AVV with HCMV-driven constructs were also used. One of them expressed a dominant form of Akt (or protein kinase B). However, this intervention was without effect on Ang II inhibition of the baroreceptor reflex, which was consistent with the outcome of pharmacological experiments (85). In summary, AVV expressing various dominant-negative proteins helped us to unravel the functional significance of eNOS in



**Fig. 3.** Determining the intracellular signaling pathway activated by angiotensin II (Ang II) to inhibit the baroreceptor reflex. Two possible pathways were tested (**A,B**). Using adenoviral gene transfer (and pharmacological antagonists—*see ref. 10*) to disrupt pathway A prevented the Ang II-induced attenuation of the baroreceptor reflex. Two adenoviruses were used to express dominant negative proteins to block Gq (AVV 1) and endothelial nitric oxide synthase (eNOS) (AVV 2). In contrast, reducing reactive oxygen species (ROS) by overexpressing catalase (AVV 3) or expressing a dominant-negative to block protein kinase B activity (Akt; AVV 4) were unable to prevent the baroreceptor reflex attenuation by Ang II. It has been hypothesized that pathway A is responsible for the Ang II-induced activation of eNOS. (Data taken from refs. 10 and 32.)

the NTS for long-term control of baroreceptor reflex sensitivity and arterial pressure in conscious normotensive and hypertensive animals, as well as the intracellular signaling pathway that links AT<sub>1</sub> receptor to baroreflex inhibition.

**Three Methodological Aspects of Using Dominant-Negative Proteins**

First, expression of the dominant-negative protein might give a more accurate portrait of the function of endogenous molecules than their overexpression, provided that the dominant-negative protein is specific and potent. Indeed, if a biologically active transgene appears in different cellular phenotypes that normally do not express it (as is the case with most overexpression experiments), this may give the wrong idea about the function of this transgene. In contrast, a dominant-negative protein is not supposed to have any functional

consequence in cells other than to block the activity of the native protein, if it is present. Theoretically at least, it could be argued that the use of dominant-negative proteins should be somewhat more selective, and any effect more reliably interpreted than the responses that occur following overexpression of proteins in the “gain-of-function” experiments.

Second, as mentioned previously, we have used largely AVV with the HCMV promoter, with one exception being the EF1 $\alpha$ -driven construct. We want to draw attention to this issue because this is an example of a case where viral transgenesis differs radically from the use of a pharmacological blocker: expression of a transgene might result in its inadvertent targeting of a subset of cells with a specific phenotype. As mentioned previously, we have recently found that HCMV-driven expression is not as ubiquitous in the brain as is sometimes thought (27). A striking result was obtained in experiments in organotypic brainstem slice cultures co-transfected with two AVV at the same time: the first AVV contained an HCMV-DsRed expression cassette (DsRed is a far-red shifted fluorescent protein from coral), whereas in the second AVV expression of EGFP was controlled by the PRSx8 artificial promoter, which is highly active in noradrenergic, adrenergic, and some vagal preganglionic neurons. In several brainstem noradrenergic cell groups, no colocalization of DsRed and EGFP could be detected in numerous (>50) cells using spectral confocal microscopy (for details, *see* 86). In contrast, co-infection with HCMV-DsRed AVV and HCMV-EGFP AVV resulted in more than 90% of double-labeled cells (many of which had the characteristic glial morphology; unpublished observation). These results were consistent with the outcome of *in vivo* experiments where noradrenergic neurones in the locus coeruleus did express EGFP, but did not express DsRed after injection of a mixture of PRSx8-EGFP and HCMV-DsRed AVV, although red fluorescent cells were visible outside this nucleus (27). Interestingly, HCMV-EGFP AVV caused robust expression in brainstem cholinergic motor nuclei of the hypoglossal nerve (Fig. 1D,E). The ability of AVV with the HCMV promoter to efficiently target glia has also been documented before both *in vivo* (87) and in acute brain slices (88). In addition, AVV with HCMV-EGFP can transduce local vascular endothelium after brainstem microinjections *in vivo* (89). Therefore, it is possible, and even likely, that HCMV-controlled vectors discriminate against some cellular phenotypes in favor of others and the physiological outcome of these experiments may be the result of expression in nonneuronal cellular compartments, such as the local vascular cells or glia.

A dominant-negative approach has also been employed to analyze the role of Rho/Rho kinase pathway in the NTS (6). Rho is a small GTPase, present in many cells, that can be activated by a number of extracellular signals. In the GTP-bound state, Rho binds to numerous intracellular targets to activate them. One such target is Rho kinase. The dominant-negative Rho kinase bears mutations in its adenosine triphosphate-binding domain and effectively inhibits the Rho-mediated phosphorylation of its numerous downstream targets. AVV with HCMV promoter to drive expression of this dominant negative protein were injected into the NTS of both WKY and SHR, from which blood pressure was measured using radiotelemetry. In both strains a transient drop in heart rate and blood pressure was observed between d 4 and d 7, but this was more dramatic in the SHR. The authors also found that in the SHR, Rho redistributes to the plasma membrane from the cytosol, and that phosphorylation of one of the downstream targets of Rho kinase in SHR is enhanced. In addition, a small but statistically significant decrease in both eNOS and nNOS protein was found after Rho-kinase dominant-negative protein expression in the NTS. In this study, an AVV with the HCMV promoter was used (6). Thus, based on our

evidence (27), it can be predicted that only a small minority of targets transduced by this vector in the NTS could be neurones. What then could be the cellular target of the Rho-kinase dominant-negative expression in the NTS described by Ito et al. (6)? Clearly, in contrast with the previously discussed eNOS overexpression experiments, the Rho-kinase dominant-negative could only act on Rho signaling within the same cells expressing the transgene. Given that the effect was very transient (3 d only), it is possible that the local endothelial cells were responsible for it; these cells can express transgenes by injection of AVV with HCMV promoter in the brain (89). They are exposed to the immune system and are likely to be rapidly eliminated as a result the residual expression of the adenoviral proteins. A change in eNOS expression reported in that study could be consistent with this suggestion (6).

In conclusion, the examples presented illustrate the usefulness of expressing dominant-negative proteins in unraveling neuronal mechanisms in central regulation of cardiovascular function. These have allowed identification of ligand-mediated intracellular cascades *and* the simultaneous physiological consequences of perturbing such pathways on blood pressure regulation. There is no doubt that viral gene transfer coupled with radiotelemetry provides a powerful tool for the cardiovascular neuroscientist. However, further refinement of viral constructs is required. As exemplified by the AVV with HCMV-driven transgenes, caution is required when interpreting data and ascribing functional effects to a specific cell type within the brain.

### ***Tracking Cardiovascular Circuits Using Retrograde Viral Transfection***

Certain types of viruses (e.g., the pseudorabies virus) can be used to track neuronal pathways as a result of their retrograde transport and ability to express transgenes, such as EGFP, in connected central neurones, which presumably form a neuroanatomical circuit. Irnaten et al. (13) used the Bartha strain of swine pathogenic pseudorabies virus in a study on cardiorespiratory networks. Pseudorabies virus is an attenuated replicating pathogen, and is therefore different from all other vectors mentioned in this chapter because it is *not* replication-deficient. It enters neurones via their peripheral axonal terminals and invades the soma by retrograde transport. Once in a neuronal soma, the virus replicates within a few hours, becomes highly concentrated in dendritic processes, and then leaves its host at sites of synaptic contact. It is thought that glial processes limit the lateral spread of virions to unrelated nerve cells and contribute to a predominantly transsynaptic pattern of viral spread (*see* refs. 90,91). Irnaten et al. (13) engineered a Bartha virus mutant to express EGFP using an endogenous gG promoter present in that virus. Two days after the injection of the virus into the pericardial sac of rat pups, EGFP fluorescent cardiovascular neurones could be identified in the nucleus ambiguus. However, an adjacent population of motoneurones with their axons in the superior laryngeal did not express EGFP. Electrophysiological analysis revealed that the EGFP-expressing neurones could generate action potentials and had currents similar to those in nearby nontransfected neurones. If pups were sacrificed 3 d after pericardial injections, EGFP labeling was also found in the periambigular area and NTS. However, *in vivo* analysis of transfected pups revealed that viral infection caused a clear shift in the baroreceptor reflex input–output curve measured at d 3, suggesting a change in the functional state of the reflex pathway (13). In conclusion, this viral approach allows clear identification of cardiac vagal motoneurones, and in this regard is similar to that achieved using conventional fluorescent retrograde tracers, such as rhodamine beads. It appears, however, that interneurones antecedent to the motoneurones are much more difficult to study. It is

highly unlikely that the infected neurones retain normal neurophysiological characteristics for any length of time because of the detrimental effects of rapidly replicating virus.

AVV can also be used for retrograde tracing, but they do not pass transynaptically. Moreover, as AVV do not cause any deleterious effects in the neurones they infect, they can also be used to coexpress a functional protein along with a fluorescent reporter. For example, cardiac parasympathetic preganglionic neurones in nucleus ambiguus were retrogradely transfected with AVV after injection of this virus into the pericardial sac of P4–P10 rat pups (12). The AVV contained two transgenes in a head-to-head orientation, both under control of the HCMV promoter. One of these cassettes expressed EGFP, and the other—the  $\epsilon$ -subunit of GABA<sub>A</sub> receptor. Two to four days after viral injection medullary slices were prepared. Retrogradely transduced fluorescent neurones could be identified and their electrophysiological characteristics studied using the patch clamp technique. It was found that expression of the  $\epsilon$ -subunit in cardiac vagal motoneurones blocked the pentobarbitone-induced potentiation of GABAergic currents.

Finally, and as mentioned previously, we have developed a method for selective retrograde transduction of subsets of brainstem noradrenergic neurones which may be used to analyze their function (27).

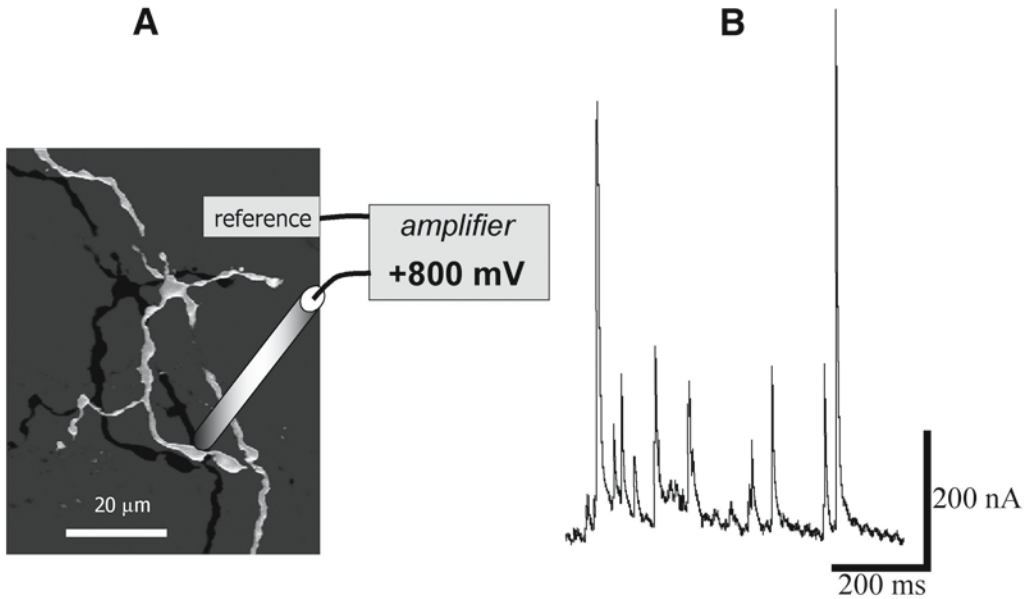
### *Future Potential Applications of Viral Gene Transfer*

There may be several other applications of viral gene transfer in studies of central cardiovascular control; we wish to describe two. First, our laboratory recently demonstrated direct recording of transmitter release from individual visualized varicosities of brainstem noradrenaline-containing neurones. An AVV was constructed to express EGFP under control of the PRSx8 promoter, which is highly active in noradrenergic neurones (*see* previous section). In slice cultures, catecholaminergic neurones displayed characteristic beaded axons that were readily identifiable using either conventional fluorescent or confocal microscopy (Fig. 1F). Moreover, these axons could be traced for hundreds of microns. Using fine carbon electrodes, microamperometric recordings of noradrenaline release from these varicosities can be made (Fig. 4). Thus, this novel approach will allow for the first time a study of the modulation of noradrenaline release from characterized central neurones of normotensive and hypertensive rat strains. Second, the “vigilant vector” introduced by Phillips and colleagues (92) has a number of unique characteristics. It has both a cardiac myocyte-specific promoter (a truncated version of MLC2v) and an enhancer, with the hypoxia response element operating within a single AAV. This construct drives expression of antisense RNA to the AT<sub>1</sub> receptor in the heart in hypoxia-inducible manner. The motivation for such a construct was based on the problem of myocardial ischemia, which initially is a progressive asymptomatic condition. Following a hypoxic episode in the heart, AT<sub>1</sub> receptor antisense expression could help alleviate consequences of ischemia, because this receptor contributes to this condition. Such vigilant vectors have not yet been applied to the central nervous system, but it is conceivable that expressing transgenes to depress AT<sub>1</sub> receptors in specific cell types could be most beneficial in neurogenic hypertension. Cascades which link neuronal activity to c-fos gene expression could possibly be exploited to achieve inducibility as described in ref. 93.

## CONCLUSIONS

There is no question that genetic manipulation to address biological questions has recently become a universal research tool in all areas of physiology. As illustrated herein,





**Fig. 4.** Detection of noradrenaline release from visualized varicosities of A1 neurones. **(A)** Schematic to illustrate the principle of microamperometric catecholamine detection. Oxidation of the catecholamine at the preset electrode potential generates current, as shown in panel B. In these experiments, a 5- $\mu\text{m}$  carbon fiber electrode was positioned directly on the visualized enhanced green fluorescent protein (EGFP)-fluorescent axonal varicosities. **(B)** A current trace illustrates bursts of vesicular noradrenaline release from a single varicosity.

viral vectors offer an efficient method of gene transfer, and can be used to perform a variety of experiments in order to address mechanisms of central cardiovascular control. So far, mainly AVV and LVV have been used in these studies, but AAV also has very attractive features. All available viral vectors target (albeit sometimes inadvertently) the transgene to one or more cell types in the brain (glia, different components of blood vessels, various neuronal phenotypes). This occurs at both the transductional (e.g., at the stage of viral entry into the cells) and transcriptional levels (e.g., via the mechanisms which control gene expression), and needs to be taken into account when interpreting the functional outcome of such experiments. It is expected that in the near future, viral vector production will become faster and cheaper, and that viruses will be completely nonimmunogenic, with targeted cellular tropism in which expression is controllable and suitable for both *in vivo* and *in vitro* applications. It may also become important to further refine protocols for production of large quantities of vectors, such as LVV, for systemic applications. Finally, further research should focus on developing short and powerful promoters suitable for directing virally mediated expression to selected populations of cells. Viral gene manipulation in the brainstem has already been successfully used to identify mechanisms relating to cardiovascular control, as well as the alterations that occur and that may contribute to hypertension. In this type of research, viral gene transfer is particularly valuable because it is fully compatible with other established conventional experimental strategies, such as hypertensive rat models and long-term telemetric monitoring of blood pressure.

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## Vigilant Vectors

### *Intelligent Gene Vectors for Cardioprotection in Myocardial Ischemia*

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*M. Ian Phillips, PhD, DSc and Yi Tang, PhD*

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#### SUMMARY

The study describes the development of a vigilant vector for protection against myocardial ischemia. The concept is a vector that can be systemically injected that waits in the heart for an ischemic attack. The vector then switches on cardioprotective genes specifically in the heart to save the cells from apoptosis and maintain cardiac performance. Using the adeno-associated virus (AAV), we have developed a double vector system (a sensor and an effector vector), that has a gene switch responsive to hypoxia, and a myosin light chain 2 ventricular (MLC-2v) promoter so that expression only occurs in the heart. The gene switch causes expression of therapeutic genes, such as heme oxygenase 1 (HO-1) and super oxide dismutase (SOD), to provide cardioprotective effects. The sensor AAV contains an oxygen-dependent domain (ODD) of hypoxia-inducible factor-1 (HIF-1 $\alpha$ ). The vector is switched on by low oxygen (<1%) and switched off by normoxia. To amplify the cardioprotective effect, an amplification system is added to the gene switch. This system consists of the yeast gene, GAL4, together with the NF $\kappa$ B protein P<sub>65</sub>. The MLC-2v promoter drives the expression of a fusion protein of GAL4/p65 exponentially with low oxygen. This fusion protein binds to the effector component of the amplification system in a second AAV or plasmid. The effector

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AAV has a GAL4 upstream sequence-binding site. Binding to this site by the fusion protein causes the expression of cardioprotective genes at a rate that increases exponentially as oxygen levels are reduced. This has been successfully tested *in vitro* in myocardial cells, including both embryonic prenatal and adult rat cardiomyocytes; and *in vivo* in mouse myocardial ischemic hearts.

**Key Words:** Vigilant vector; myocardial ischemia; gene switch; AAV vector; cardio-protection MLC-2v promoter.

## INTRODUCTION

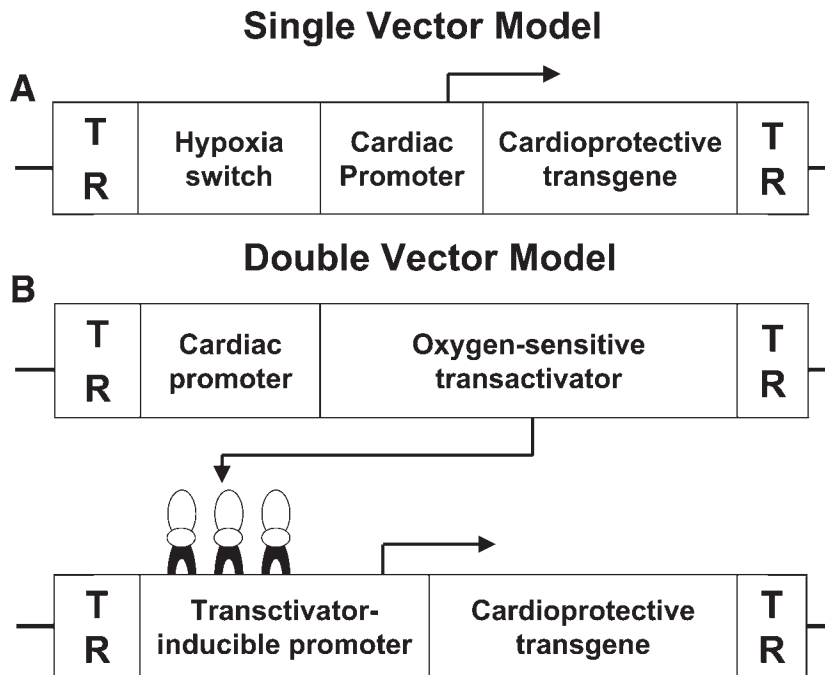
Vigilant vectors are intelligent in that they are designed to switch on or off (1). This is an advance over current gene therapies where a gene is used to replace a mutated gene but is secreted constitutively. Many life-threatening and chronic diseases create physiological signals that could be used to switch on therapeutic genes. We are developing a gene therapy approach in which a systemically injected vigilant vector waits for these signals and switches genes on to protect specific tissues with high amplification. The concept of a vigilant vector requires four components (Fig. 1). The first component is a safe and stable vector that can be administered by systemic injection and can express transgenes in a particular organ or tissue. The adeno-associated virus (AAV) vector is safe and stable for this purpose. The second component is a reversible gene switch which acts as a biosensor that can detect certain physiological signals. We are developing a hypoxia switch, based on the oxygen-dependent degradation (ODD) domain of hypoxia-inducible factor (HIF-1 $\alpha$ ). The third component is a tissue-specific promoter, and we have used the myosin light chain-2 ventricular (MLC-2v) promoter for specific expression in the heart. The fourth component is an amplification system. For this we have developed a double-plasmid/vector system based on the yeast GAL4, and human transcriptional activator p65 to produce a transactivating fusion protein that binds to a GAL4 activation sequence in an activating plasmid that then expresses high levels of cardioprotective genes. Repeated bouts of hypoxia in coronary artery disease lead to silent or overt myocardial tissue damage (2). In diabetes, the signal is high glucose; in cancer, the signals are tumor markers.

We have found that the design is not obvious. A single vector containing the gene switch promoter and transgene does not, by itself, produce enough protective genes when the gene switch is on. This may be because of interference between the switch sequences and the promoter sequences. Therefore, we have developed a double-plasmid (or vector) system to greatly amplify the transgene expression to provide the maximum protection.

To demonstrate how these components come together in an adeno-associated viral vector to make a vigilant vector, we have focused on designing the vector for use in protecting the heart during ischemia (2,3). Therefore, the gene switch senses hypoxia and the promoter is specific in the heart.

## VECTOR

For the vector, the recombinant adeno-associated virus (rAAV) is proving to be a stable, nonpathological vector (4,5). Our data and data from other studies show that a single injection of rAAV (serotype 2) expresses transgenes in tissue for at least 6–18 mo (5).



**Fig. 1.** Two models for the vigilant vector. **(A)** Single-vector model. Hypoxia switch, cardiac promoter, and cardioprotective transgene are adapted between the inverted terminal repeats (TR) of adeno-associated virus (AAV). **(B)** Double-vector model. In a single AAV, the cardiac promoter controls an oxygen-sensitive transactivator. The transactivator produces a fusion protein which binds to the inducible promoter and activates transcription of cardioprotective transgene in the second AAV.

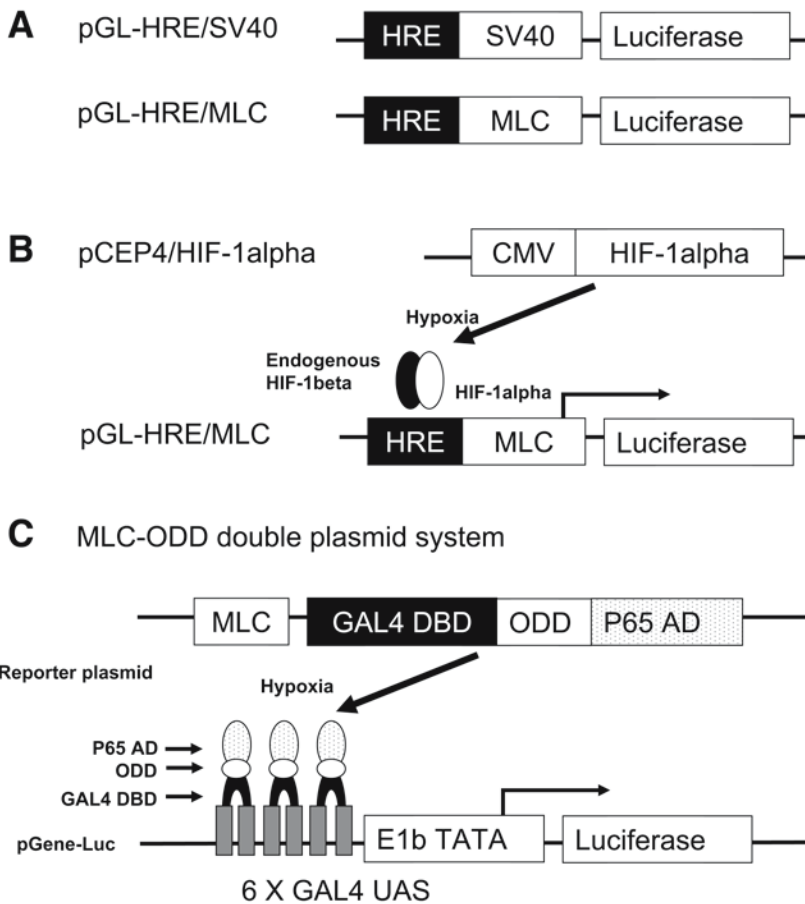
## GENE SWITCH

For ischemia, the gene switch for the vector has to sense hypoxia. Hypoxia triggers a multifaceted adaptive response in mammalian cells, which is primarily mediated by a family of transcription factor termed HIF-1 $\alpha$  and its cognate DNA recognition site, known as hypoxia response element (HRE) (6,7). By utilizing HIF-1 $\alpha$  or HRE, we have studied three models of hypoxic switch (Fig. 2).

To test the ability of HRE to induce transcription under hypoxia in cardiomyocytes, we tethered HRE to the SV40 promoter or MLC-2v (8) promoter and put them upstream of a luciferase reporter gene (Fig. 2A).

Under normal oxygen condition, the HIF-1 $\alpha$  is undetectable (7) as a result of its rapid destruction by the ubiquitin–proteasome system (9–13). But during hypoxia, HIF-1 $\alpha$  is no longer degraded, and accumulates exponentially as cellular O<sub>2</sub> decreases (12). Within the HIF-1 $\alpha$ , there is an ODD domain (amino acids [aa] 401–603) (11, 13, 14). It independently controls ubiquitin–proteasome-mediated degradation of HIF-1 $\alpha$ . The deletion of this entire region stabilized the HIF-1 $\alpha$ . Conversely, the ODD domain alone confers oxygen-dependent instability when fused to a stable protein, Gal4. Thus, the vigilant vector contains an oxygen-sensitive chimeric transactivator (GAL4/ODD/p65), which





**Fig. 2.** Three models of a hypoxia switch. **(A)** Hypoxia-response element (HRE) has been inserted upstream of either the SV40 or the myosin light chain 2 ventricular (MLC-2v) promoter to switch on luciferase expression in response to hypoxia. **(B)** Coexpression of hypoxia-inducible factor (HIF)-1 $\alpha$ -containing plasmid with the HRE/MLC-2v promoter. Under hypoxia, the HIF-1 $\alpha$  subunit is stable and activated to dimerize the HIF-1 $\beta$  subunit. This binds to the HRE, stimulating transcription from the HRE/MLC-2v promoter. **(C)** Oxygen-sensitive chimeric transactivator (GAL4/ODD/p65), which is expressed by the transactivator plasmid under the control of the MLC-2v promoter. It can accumulate under hypoxia and activate the inducible promoter containing the GAL4 upstream activation sequence in the reporter plasmid.

was constructed by inserting ODD between the yeast GAL4 DNA-binding domain and p65 activation domain.

### TISSUE SPECIFICITY

The method of using vigilant vector is to inject them systemically. Therefore, they will be ubiquitously distributed. However, by adding a tissue-specific promoter, the vector is designed to be expressed in only one tissue. To specifically express transgenes in the heart, we have used the MLC-2v promoter, which is highly specific for the heart, during both embryonic and postnatal development (8) The MLC-2v promoter is 3.0 kb, but the proximal 250-basepair (bp) sequences, which contain the TATA box and several con-

served *cis* regulatory heart factor (HF) sequences, HF-1 (HF-1a and HF-1b) (15–17), HF-2 (17,18), and HF-3, are sufficient to confer not only cardiac muscle specificity, but also ventricularly restricted expression (18–22).

What is not obvious is that we could reduce the length of the MLC-2v to 280 bp and still produce heart specificity. We tested the specificity of a 1700-kb MLC-2v promoter in rAAV *in vivo* and a 280-bp MLC-2v promoter *in vitro*.

## CARDIOPROTECTIVE GENES

There are several genes that could be considered for protection of the heart during ischemia. In previous studies, we showed that antisenses to angiotensin II type-1 receptor (23,24), antisense to adrenergic  $\beta_1$ -receptor (25), or antisense to angiotensin-converting enzyme (26) protect rat hearts from ischemia–reperfusion injury. Recent use has shown that heme oxygenase-1 (HO-1) is cardioprotective when delivered in a vigilant vector (27). HO-1 degrades the prooxidant heme and generates carbon monoxide and antioxidant bilirubin (28). Alternatively, superoxide dismutase (SOD) protects the heart against superoxide radicals generated during ischemia–reperfusion (28,29). Thus, these genes are good choices for cardioprotective transgenes in the vector.

## TRANSGENE AMPLIFICATION

It may seem obvious to use a single plasmid or vector containing the required elements. But, when testing a single vector with the gene switch for hypoxia and an MLC-2v promoter, we found that there was not the expected increase in transgene expression at 1% O<sub>2</sub>. Therefore, we developed an amplification system using two plasmids (double-plasmid system) (Fig. 3)—one plasmid or vector is the sensor, the second is the effector. The sensor contains a transcriptional activator (GAL4/p65) which consists of yeast GAL4 DNA-binding domain and p65 activation domain (30). When activated, the gene products form a fusion that binds to a GAL4 upstream activating sequence (UAS) inserted in the effector plasmid, and powerfully activates transgene transcription. We tested the amplification effect of this system on MLC-2v, SV40, and cytomegalovirus (CMV) promoters. In addition to amplification, this double-plasmid system provides a convenient platform for the regulation of gene expression in response to hypoxia by replacing the GAL4/p65 with a chimera containing the oxygen-sensitive gene switch ODD (Fig. 2C) or using HRE/SV40 enhancer/promoter to control GAL4/p65.

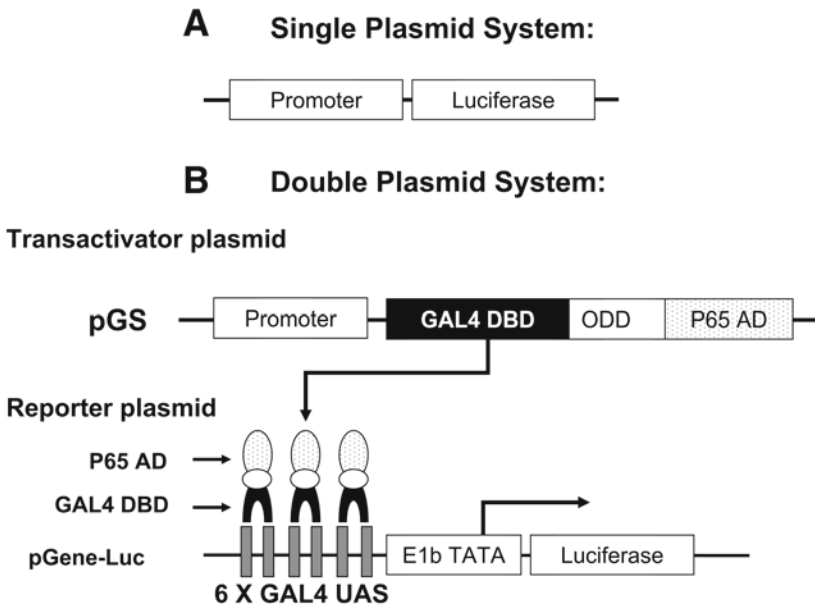
## METHODS

### *Construction of AAV-Based Plasmids*

We inserted a rat 1.7-kb MLC-2v promoter and the coding sequence of GFP between the inverted terminal repeats of AAV vector to construct pMLC-2v–GFP (2).

The plasmid, gene luciferase (pGL) with HRE and simian virus (SV) 40 promoter (pGL-HRE/SV40 [31]), was derived from pGL-SV40 (pGL2-Promoter, Promega, Madison, WI) by insertion of a 68-bp human enolase (ENO) 1 HRE sequence (–416 to –349, GenBank accession no. X16287) into the 5' flank of the SV40 promoter. The HRE sequence was also inserted into the 5' flank of the MLC-2v promoter in the pGL-MLC to generate pGL-HRE/MLC (2).

pCEP4 (a specific plasma mold from Invitrogen, Carlsbad, CA)/HIF-1 $\alpha$  (32), which contains human HIF-1 $\alpha$  cDNA sequence downstream of a CMV promoter.



**Fig. 3.** The amplification system. (A) A single-plasmid system is the traditional way to express transgene, in which a promoter drives transgene (luciferase) expression directly in one plasmid. However, this does not produce enough transgene. (B) A double-plasmid system can amplify the power of promoter based on the strong transcription activity of GAL4/p53 fusion protein. The promoters in the transactivator plasmid gene sensor (pGS) tested included SV40, HRE/SV40, cytomegalovirus (CMV), and myosin light chain 2 ventricular (MLC-2v) promoter. The reporter plasmid contains GAL4 upstream activation sequence (UAS) in front of an adenovirus E1b TATA box and the firefly luciferase reporter gene. The fusion protein binds to the GAL4 UAS and promotes an amplified transgene expression.

We have developed the use of the ODD domain as a gene switch for hypoxia. ODD (aa 394–603) (13) was amplified by polymerase chain reaction (PCR) from pCEP4/HIF-1 $\alpha$  (31) and inserted in-frame between the coding sequence of GAL4 DNA-binding domain and p53 activation domain (GAL4/ODD/p53) in plasmid gene sensor (pGS)-MLC to generate pGS-MLC-ODD. The key to the invention, which is not obvious, is the insertion of ODD between GAL4 and p53, which confers a powerful O<sub>2</sub> switch action when linked to a promoter.

### *Transgene Amplification Plasmids*

#### **SINGLE-PLASMID SYSTEM**

The single-plasmid system is shown in Fig. 3A. Three promoters have been inserted in front of the transgene, which in this case is a luciferase reporter gene. pGL-SV40 (Promega, CA) contains SV40 promoter. Plasmid myosin light chain promoter-luciferase harbors a 281-bp (–264 to 17, GenBank accession no. U26708) fragment of MLC-2v promoter. Plasmid cytomegalovirus promoter-luciferase has CMV enhancer and promoter.

#### **DOUBLE-PLASMID SYSTEM**

To activate a highly amplified transgene expression, the double-plasmid system (Fig. 3B) is used. For the transactivator plasmid, pGS-CMV expresses a chimeric transcription

factor consisting of the yeast GAL4 DNA-binding domain (aa 1–93) (33) and the human p65 activation domain (aa 283–551) (34) from nuclear factor (NF)- $\kappa$ B under the control of a CMV enhancer/promoter. The CMV promoter has been replaced with an SV40 promoter, HRE/SV40 enhancer/promoter, or 281-bp MLC-2v promoter to generate pGS-SV40, pGS-HRE/SV40, and pGS-MLC, respectively. For the reporter plasmid, pGene-Luc encodes luciferase driven by six copies of a 17-bp GAL4 UAS (35) and an adenovirus-derived E1b TATA box (36). It was derived from pGene/V5-His/lacZ (Invitrogen, Carlsbad, CA) by replacing the *lacZ* coding sequence with a luciferase cDNA. The identity of clones was confirmed by nucleotide sequence analysis.

### *Testing the Plasmids In Vitro*

A rat embryonic cardiac myoblast cell line, H9c2 (CRL1446; ATCC, Manassas, VA), was maintained in Dulbecco's modified Eagle's medium supplemented with sodium pyruvate and 10% fetal bovine serum. Cells were cultured under normoxic conditions (5% CO<sub>2</sub>, 20% O<sub>2</sub>, 75% N<sub>2</sub>, v/v) in a humidified incubator at 37°C. For hypoxic treatment, cells were put into hypoxia chambers. The chambers were connected to a tank with premixed gas (1% O<sub>2</sub>, 5% CO<sub>2</sub>, and 94% N<sub>2</sub>) at one end and to a vacuum at the other end. Hypoxia condition was achieved by evacuating and gassing the chambers for six times. Then the tightly sealed chambers were incubated at 37°C. The oxygen level in the chamber and medium is around 7.6 mmHg, which was monitored by an OxyLite probe (Oxford Optronix, Oxford, UK).

Cells were transfected at a confluence of 50–60%. Transfection was performed with LipofectAMINE (Invitrogen) according to manufacturer's protocol. pRL-CMV (Promega) or pRL-TK (Promega) coding *Renilla* luciferase was used to control transfection efficiency. Luciferase assays were performed with dual luciferase assay system (Promega). Results were quantified with a Monolight 3010 luminometer (PharMingen, San Diego, CA) and expressed as a ratio of firefly luciferase activity over *Renilla* luciferase activity.

### *Intracardiac Injection*

For intracardiac injection, the animal is lightly anesthetized with an inhalant, such as metofan or isoflurane, and placed on its back. The needle of the syringe is injected under the xiaphoid process, angled slightly left to enter the left-ventricular chamber. The syringe is drawn back until blood is seen and then the injectate is deposited directly into blood. The advantage of this method is that it utilizes direct injections that are rapidly distributed through the body. In our experience with this method of injection (37), we have not seen any toxic response or infection.

## RESULTS

### *Cardiac Specificity*

In vivo, the pGL-MLC was specifically expressed in cardiomyocytes. After transient transfection, the luciferase expression in cardiomyocytes (H9c2) is  $29.38 \pm 13.11$ -fold higher than that in rat glioma cells (C6) (2) and  $20.07 \pm 1.71$ -fold higher than that in pulmonary vein endothelial cells. The transfection efficiency in three cell lines was normalized by the expression of an internal control plasmid containing ubiquitous viral promoter (CMV or thymidine kinase promoter).

In vivo, 4 wk after a systemic injection into adult mice or 5-d-old rats, the transduction of rAAV-MLC-2v-GFP was shown by PCR of DNA in many tissues, such as spleen, liver, lung, kidney, and heart. The tissue-specific expression of GFP mRNA under MLC-2v promoter was examined by reverse transcription (RT)-PCR and was detected only in heart (2). The presence of GFP protein was further examined by immunofluorescence staining, which was apparent in the heart of the treated animal and absent in the control animal (injected with saline). GFP was undetectable in the kidney and liver of the same treated animals and controls (2).

### *Hypoxia-Inducible Gene Switches*

H9c2 cells were transfected with pGL-HRE/SV40 alone, pGL-HRE/MLC alone, or both HIF-1 $\alpha$  and pGL-HRE/MLC and then subjected to 1% O<sub>2</sub> for 24 h (Fig. 4). The luciferase expression of HRE/SV40 (Fig. 4A) construct increased by  $7.12 \pm 1.52$ -fold in cells incubated under hypoxia relative to cells incubated under 20% O<sub>2</sub>. However, pGL-HRE/MLC did not show significant hypoxic induction. In the presence of overexpressed HIF-1 $\alpha$ , HRE/MLC showed three- to fourfold increases under hypoxia (Fig. 4B).

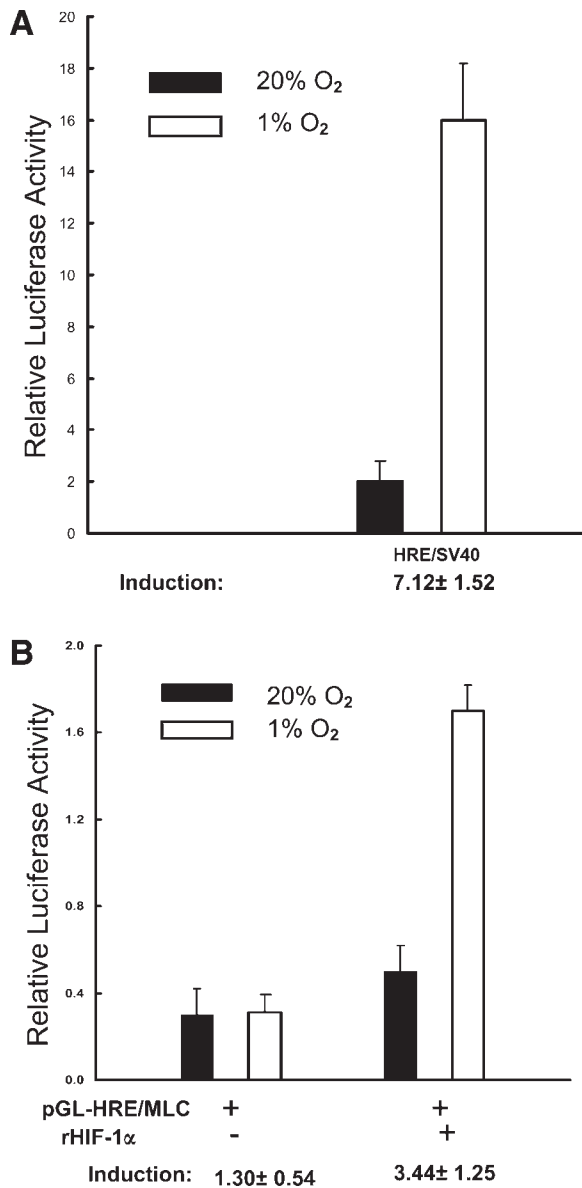
The MLC-ODD double-plasmid system was tested under 1% O<sub>2</sub> for 24 h (Fig. 5). In H9c2 cells, this system showed  $5.75 \pm 1.38$ -fold increase of expression. This system worked even better in the primary culture of adult rat cardiomyocyte, with the hypoxia induction ratio at  $17.89 \pm 4.85$ . Without ODD, GAL4/p65 fusion protein did not increase reporter expression. Therefore, the ODD was acting as a biosensor for low oxygen.

### *The Amplification Effect*

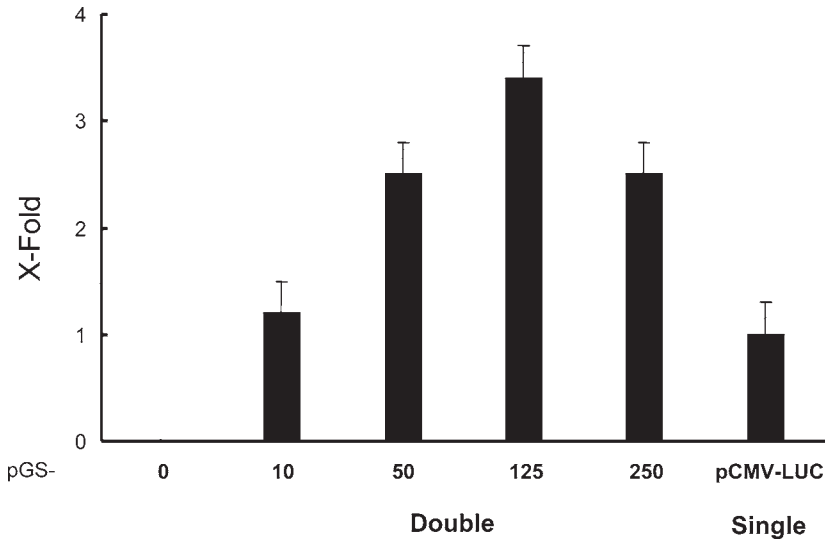
For a strong promoter, such as CMV, the double-plasmid system could increase the power of CMV promoter up to  $3.52 \pm 0.33$ -fold over that of the single-plasmid (Fig. 5). For a relatively weak promoter, such as SV40, there was a  $410.56 \pm 84.42$ -fold increase (3). For a tissue-specific promoter, such as MLC-2v, the increase was  $346.08 \pm 22.50$ -fold without compromising cardiac specificity expression. Although by adding a higher dose of transactivator plasmid we could further increase the expression level of the MLC double-plasmid system, the tissue specificity was decreased. The double-plasmid system also effectively increased the level of HRE/SV40 promoter up to  $412.79 \pm 185.27$ -fold at 1% O<sub>2</sub> and  $205.35 \pm 65.44$ -fold at 20% O<sub>2</sub> and still maintained its hypoxic induction ability (3). The HRE/SV40 double-plasmid system started increasing expression at 4 h; this increase is reversible (Fig. 6).

### *Time of Onset*

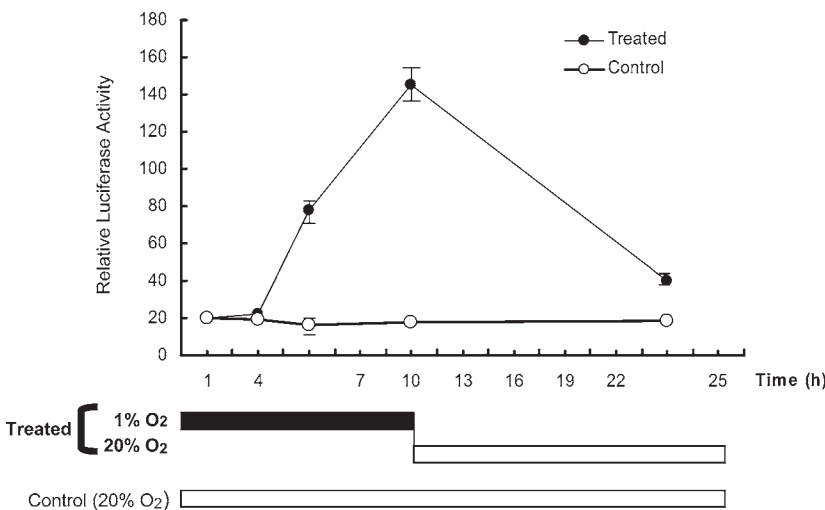
Although we do not know the time of onset of gene activity in vivo, we have tested time-of-onset in vitro. Careful measurements of O<sub>2</sub> levels in the cultures have revealed that it takes 2.6–3.8 h after O<sub>2</sub> has been reduced from 20 to 1% in a closed chamber to reach 1% in the culture medium. The reporter gene in vector in this cell culture system was significantly higher at 4–6 h. Therefore, subtracting the time to reach 1% in the media, the activation of the system is about 0.2–1.4 h. This indicates that using Western blotting of transgenes such as HO-1, we can detect expression within 10–30 min after ligation of the left anterior descending coronary artery in mice (27). The time for therapeutic genes to act is within this time frame, except in absolute anoxia.



**Fig. 4.** The hypoxia-inducible ability of hypoxia-response element (HRE) containing gene switch. **(A)** HRE can switch SV40 promoter on in response to 1% O<sub>2</sub>; 2  $\mu$ g/well pGL-HRE/SV40 was transfected into H9c2 cells along with 100 ng/well pRL-TK control plasmid in 60-mm dishes. Duplicate plates were incubated in either 20 or 1% O<sub>2</sub> for 24 h before preparation of cell lysates. Results are expressed as a ratio of firefly luciferase activity over *Renilla* luciferase (relative luciferase activity). The ratio of relative luciferase activity in cells at 1% O<sub>2</sub> compared to 20% O<sub>2</sub> was also calculated to determine induction by hypoxia (mean  $\pm$  standard deviation (SD)  $n = 3$  independent experiments). **(B)** HRE/myosin light chain 2 ventricular (MLC-2v) enhancer/promoter alone could not augment luciferase expression under hypoxia. However, in the presence of rHIF-1 $\alpha$ , HRE/MLC-2v could increase luciferase expression by three to fourfold in response to hypoxia. H9c2 cells were cotransfected with 2  $\mu$ g/well pGL-HRE/MLC and 100 ng/well pRL-TK, in the absence or the presence of recombinant hypoxia-inducible factor (rHIF)-1 $\alpha$ . The cells were exposed to 1 or 20% O<sub>2</sub> for 24 h (mean  $\pm$  SD;  $n = 4$  independent samples).



**Fig. 5.** The double-plasmid system amplified the power of the cytomegalovirus (CMV) promoter. For double-plasmid system transfection, H9c2 cells seeded in six-well plates received 125 ng/well control pRL-TK, 250 ng/well reporter plasmid (pGene-Luc), 0–250 ng/well transactivator plasmid (pGS-CMV), and various amounts of empty vector such that all cells received a total of 625 ng plasmid per well. The transfection of 250 ng/well pCMV-Luc served as the single plasmid system. The relative luciferase activity from double-plasmid transfection was normalized to that obtained from the single-plasmid system (X-Fold) (mean ± SD; *n* = 3 independent samples).



**Fig. 6.** The switch on-and-off function of the hypoxia-responsive element (HRE)/SV40 double-plasmid system. H9c2 cells were transfected with 10 ng/well pGS-HRE/SV40 and 2 µg/well pGene-Luc. The cells in the treated group were first exposed at 1% O<sub>2</sub> for 10 h and then returned to 20% O<sub>2</sub> for another 14 h. Thus, the luciferase expression was first increased during the hypoxic period, then dropped down after reoxygenation. The cells in the control group were continuously incubated at 20% O<sub>2</sub> (mean ± SD; *n* = 3–6 independent samples).

## DISCUSSION

Current gene therapy strategies insert genes, but the expression of transgenes is constitutive. This produces an unphysiological expression pattern of protein production, including inappropriate downregulation of effector systems, cellular toxicity, and pathophysiology (38,39). Therefore, being able to switch a vector on or off is a highly desirable aspect for gene therapy. Several inducible gene expression systems have been proposed. These systems are usually composed of two or three co-expressed vectors. One vector (in some cases there are two) expresses a chimeric transcription factor, which functions as a gene switch. This switch is regulated by the administration of a small molecule. The other vector contains reporter gene downstream of the switch-controllable promoter. The systems are as follows. (1) The *tet* on–off system, a tetracycline (*tet*)-regulated system, is either a *tet*-repressible or a *tet*-activating system. In the presence of the antibiotic tetracycline—a *tet*-repressor containing transactivator (tTA) does not bind to a tTA-dependent promoter, and prevents transcription. By withdrawing *tet*, the system is turned on (40). In the *tet*-activating system, the transactivator contains a mutant *tet* repressor that requires doxycycline, a *tet* derivative, for specific DNA binding. Thus, the system is turned on in the presence of doxycycline and turned off without doxycycline (41). (2) The ecdysone-inducible system (42) uses three plasmids. Two plasmids express retinoid X receptor and ecdysone receptor/VP16 fusion protein. Ecdysone binds to and activates the ecdysone receptor/VP16. The activated fusion protein and retinoid X receptor will heterodimerize and bind to ecdysone response element in the presence of muristerone A, which is a synthetic analog of ecdysone, to transactivate transcription. (3) The antiprogestin-regulated gene switch is a transactivator consisting of the truncated human progesterone receptor ligand-binding domain in the middle of the VP16 transactivation domain and the yeast GAL4 DNA-binding domain. This fusion protein activates GAL4 binding site-contained promoter in response to progesterone antagonists (43), such as RU486/mifepristone (46). (4) The dimerization-based gene switch system co-expresses two fusion proteins, one consisting of FKBP and DNA-binding domain, and the other containing FRAP and the transactivation domains (45). These two fusion proteins are associated by rapamycin through FKBP and FRAP. The heterodimeric protein complex activates transcription from an inducible promoter.

The weakness of all of these regulatable systems is that they need exogenous drugs as inducers. Not only does this produce unwanted side effects, but it also defeats the purpose of gene therapy, which is to be highly specific and nontoxic. For example, tetracycline deposits in bone and stains teeth (46). Long-term administration may lead to bacteria resistance. RU486/mifepristone is a progesterone antagonist (43). Rapamycin has growth-inhibitory and immunosuppressive effects.

Therefore, the vigilant vector is designed to respond directly to endogenous pathophysiological signals, such as hypoxia, and to switch off when normotoxia returns without any drug being given. HRE alone as a hypoxic gene switch, fused to either a similar virus (SV40) promoter (31,47) or a CMV promoter (48), has been reported to successfully increase the gene expression in response to hypoxia. We have obtained similar positive results when we tested HRE/SV40 promoter in cardiomyocytes (3). However, under hypoxia, HRE alone was not able to upregulate transcription from a tissue-specific promoter, MLC-2v promoter (2), in cardiomyocyte cultures. Overexpressed HIF-1 $\alpha$  could help HRE to regulate the MLC-2v promoter, but the expression level was quite low. We designed the oxygen-sensitive (ODD) transactivator and put it under the control of



the MLC-2v promoter. This has proven effective in both sensitivity to O<sub>2</sub> and heart under hypoxic conditions, such as myocardial ischemia (27).

According to the results of other groups (49) and ours, tissue-specific promoters are relatively weak compared to nonspecific promoters, such as CMV. The challenge is to increase the promoter strength without losing specificity. Several methods have been employed in the attempt to circumvent the power limitation of the MLC-2v promoter. An SV40 enhancer can increase the activity of MLC-2v promoter by at least 10-fold, but it decreases its cardiac specificity (50). Although three copies of the HF-1, HF-2, and HF-3 elements did not compromise the specificity of the MLC-2v promoter (2), there was only a threefold increase in activity. Overexpression of HF-1 $\beta$  leads to just a two- to threefold increase in the activity of the MLC-2v promoter (2). The double-plasmid method dramatically increased the power of the MLC-2v promoter by more than 300-fold and still maintained its specificity. This method could be applied to other tissue-specific promoters.

Our strategy needs two plasmids to cooperate, and in the context of rAAV, it will be a double-virus system. This raises the question of whether two viruses can enter the same cell. Several methods using two rAAVs to overcome its size limitation provide supporting evidence for our strategy. Relying on the intrinsic property of rAAV to undergo intermolecular concatamerization (51,52), one approach (53) has used a vector encoding multiple enhancer sequences to increase the transgene expression in a second, co-infected transgene-containing vector. The other option (54) is to split a large gene into two parts and separately package them into two individual rAAV vectors. After being co-infected into target cells, these two rAAV vectors form head-to-tail heterodimers and restore the coding sequence of the transgene after RNA splicing. Therefore, there are solutions for two rAAVs to enter the same cell and work together, should the simple empirical test of injecting two vectors at the same time prove inadequate.

The cardioprotective genes listed in the introduction protect ischemic heart through different mechanisms. We have used HO-1 for ischemia (27) but, to provide full protection, cotransfer of multiple protective genes may be necessary. For example, a rat model of Parkinson's disease coexpression of the L-dopa synthesizing enzyme, tyrosine hydroxylase, and its cofactor synthetic enzyme, GTP-cyclohydrolase-1, by rAAV provided substantial functional improvement (55). However, the effects after delivery of the tyrosine hydroxylase gene alone have been disappointing. Thus, the double virus system could be expanded to a triple (or more) virus system, with one transactivator virus expressing a strong oxygen-sensitive regulator, and several other viruses carrying different protective genes under the inducible promoter.

The concept of a vigilant vector has broad applicability. By switching the tissue-specific promoters, gene switches, and protective genes, this concept can be developed and applied generally to a number of other disease states. Among these are diabetes type 1, in which glucose would switch on preproinsulin genes, cancer where tumor markers could switch on antigrowth and antiangiogenic genes; and stroke in which hypoxia could switch on tPA. One could further imagine that a vigilant vector could lie in wait to protect against the cellular effects of biological warfare attacks, inhibiting, for example, the uptake of anthrax toxin.

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## Gene Therapy for Cerebral Arterial Diseases

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### SUMMARY

Although there has been steady progress towards cardiovascular gene therapy in humans, gene therapy for cerebrovascular disorders is still in its infancy. Several major steps, including gene transfer to cerebral arteries and alteration of gene expression, have been taken. There are several promising targets for cerebrovascular gene therapy, such as prevention of cerebral vasospasm after subarachnoid hemorrhage, stimulation of formation of collateral vessels to ischemic brain, and treatment of atherosclerotic lesions in carotid arteries. Some major obstacles, however, must be overcome before cerebrovascular gene therapy can be clinically used in humans. A key to cerebrovascular gene therapy is the development of safe and effective vectors for gene/nucleotide delivery. In addition, advances in understanding the biology of diseases and vectors will be of great value.

**Key Words:** Gene therapy; adenoviruses; cerebral arteries; carotid arteries; cerebral vasospasm; collateral circulation; carotid stenosis.

### INTRODUCTION

Since the first successful experiments of *in vivo* gene transfer to blood vessels were reported in 1990 (1), many investigators have explored gene therapy for cardiovascular diseases. Results have been reported in several clinical trials of gene therapy for cardio-

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vascular diseases, including coronary artery disease and limb ischemia (2–4). In relation to cerebrovascular disorders, however, no clinical trials for gene therapy have been reported yet.

Previously, we discussed the possibility of cerebrovascular gene therapy (5), and suggested several therapeutic targets including (1) prevention of cerebral vasospasm after subarachnoid hemorrhage (SAH), (2) stimulation of collateral blood flow to ischemic regions of the brain by angiogenesis, and (3) treatment of atherosclerotic lesions of extracranial cerebral arteries by inhibition of thrombosis or proliferation of lesions, stabilizing plaques, and prevention of restenosis after angioplasty. Recent studies using animal models demonstrate the feasibility of gene therapy for some of these targets. Some major obstacles, however, must be overcome before clinically applied to humans.

Here, we summarize the current status of studies aimed at cerebrovascular gene therapy, and discuss future directions and application in humans. Two different targets for cerebrovascular gene therapy are cerebral vasculatures and parenchymal cells of the brain. In this review, we focus on gene therapy targeted to cerebral arterial diseases.

### MINIMUM REQUIREMENTS FOR CEREBROVASCULAR GENE THERAPY

The essence of gene therapy is the correction of pathological molecular mechanisms of diseases by alteration of gene expression in a target organ or cells. Although there have been setbacks in establishing gene therapy in patients (6,7), potential advantages, such as specific and prolonged effects provided by the manipulation of specific genes, and utilization of gene expression mechanisms of the host, would make gene therapy an attractive alternative to pharmacological therapy.

For establishment of cerebrovascular gene therapy, there are several minimal requirements: first, efficient means for alteration of gene expression must be readily available. Second, access to cerebral arteries must be provided by devices or procedures that are applicable to patients. Third, alteration of gene expression must produce therapeutic effects with minimal effect on normal functions of cells and organs. Fourth, appropriate targets of treatment must be selected in terms of mechanisms and temporal characteristics of pathological process. In other words, mechanisms of the pathogenesis of target diseases must be known at the molecular level, and the time course of the effects of gene therapy must fit within the “therapeutic time window” of target diseases. Lastly, safety and ethical issues must be resolved before its clinical use in humans (8).

### ESSENTIAL TOOLS FOR ALTERATION OF GENE EXPRESSION

Basic strategies for gene therapy consist of (1) expression of deficient genes or “reconstitution,” (2) overexpression of therapeutic genes, and (3) downregulation of pathogenic gene expression. These “therapeutic” alterations of gene expression are achieved by delivery of genes or nucleotides with therapeutic potency into an organ or cells.

Vectors have generally been used for the delivery of genes or nucleotides into blood vessels (9), because uptake of “naked” DNA into the vascular wall is very limited. Replication-deficient recombinant adenoviruses are widely used for delivery of genes, both in experimental gene transfer to cerebral blood vessels and in some clinical trials of cardiovascular gene therapy (9,10). In contrast with retroviral vectors, both dividing and nondividing cells are efficiently transfected with adenoviral vectors, which is a major

advantage of the vectors in transfecting quiescent vascular cells (11). Adenoviral vectors, however, readily provoke the host's inflammatory and immune responses, with damage to the vascular wall and early loss of transgene expression (12–14). To reduce immune responses in vascular tissue, modified second- and third-generation adenoviral vectors have been produced, but they still have substantial immunogenicity (15,16).

Several studies have used adeno-associated viruses (AAVs) as an alternative for adenoviral vectors (17,18). AAV vectors are characterized by low toxicity and incorporation of delivered DNA into the host's genomic DNA, resulting in longer-term expression of transgene production. The small size of transgene capacity and difficulties in large-scale production and purification, however, are limitations for use of the vectors in gene therapy (19).

In terms of the safety of vectors, nonviral, cationic liposomal vectors are advantageous over viral vectors, because liposomes facilitate cellular DNA uptake with no substantial toxicity (20,21). In addition, liposomal vectors are capable of carrying large-sized DNA. Liposome-mediated gene transfer has been used in vascular tissue *in vivo* (9), although efficiency of transfection to vascular tissue is low in comparison with virally mediated gene transfer (22,23).

As an alternative to gene transfer of therapeutic molecules, administration of oligodeoxynucleotide (ODN) has been used for downregulation of target genes in the cardiovascular system (24). This method includes techniques utilizing inactivation of mRNA with antisense or ribozyme ODNs, and inhibition of mRNA expression with “decoy” ODNs that interact with transcription factors. This strategy is attractive because it can potentially alter the initial steps in the chain of pathological changes. There also may be problems, however, with low transfection efficiencies, short half-lives, and toxicity of ODNs (24,25).

At present, adenovirus-mediated gene transfer appears to be the most promising method of gene therapy for cerebral arterial diseases. Development of alternative approaches may be extremely useful for safe and more effective gene therapy.

## ACCESS TO CEREBRAL ARTERIES

For gene therapy, the most important characteristics of cerebral arteries that must be addressed are that the arteries supply a tissue that is very susceptible to ischemia, and that the function of specific regions within the brain is unique. Thus, it is essential to avoid ischemia, even in small regions of the brain. The first successful *in vivo* gene transfer to blood vessels was achieved in segments of the iliofemoral artery of pigs by intraluminal administration of a retroviral vector or liposomes containing a reporter gene, with interruption of blood flow for 30 min using a double-balloon catheter (1). Even in the carotid artery of certain animals, *in vivo* gene transfer can be achieved by the same approach, which usually requires interruption of blood flow for 10–30 min (for examples, *see refs. 13,23,26*). This method, however, may produce ischemic damage to the brain in humans, especially in patients who are at risk for cerebral ischemia.

Vectors or ODNs can be administered from the outside of vessels in order to circumvent the requirement for interruption of blood flow, which is usually needed for the intraluminal approach. For the intracranial circulation, transfection of vascular and perivascular cells can be achieved by injection of vectors or ODNs into cerebrospinal fluid (CSF). For example the injection of an adenoviral vector containing a reporter gene into the cisterna magna of rats effectively transfected cells in adventitia and perivascular



**Fig. 1.** Adenovirus-mediated gene transfer of bacterial  $\beta$ -galactosidase ( $\beta$ Gal) to cerebral arteries. Rat brain was examined histochemically after intracisternal injection of adenovirus encoding  $\beta$ Gal. Expression of  $\beta$ Gal (dark staining) was seen on the ventral surface of the brain, especially along major cerebral arteries. (Reprinted with permission from ref. 27.)

tissue surrounding cerebral arteries on the surface of the brain (27) (Fig. 1). Selective gene transfer to specific arteries is difficult by this method, but widespread transfection may be advantageous for treatment of some types of cerebrovascular disorders, such as cerebral vasospasm after SAH (*see* Subheading entitled “Cerebral Vasospasm After SAH”). The same technique has been applied to adenovirus-mediated gene transfer in other species including primates (28–31). In several studies, ODNs were also administered via the cisterna magna, and found to be distributed in all layers of intracranial cerebral arteries (25,32).

For the extracranial carotid artery, injection of vectors into the periarterial sheath can be used for perivascular gene delivery (33). In addition to avoiding interruption of cerebral blood flow, there appears to be less inflammatory response in the vascular wall than with the intravascular approach (34). Perivascular gene/nucleotide delivery to the carotid



artery has also been demonstrated by use of the “paintbrush” technique for adenoviral vectors (35) or by placement of slow-releasing polymers containing ODNs or plasmid cDNAs (36,37).

In addition to the perivascular approach, recent development of a new method utilizing a contrast-enhanced ultrasound technique provides intraluminal delivery of ODNs or plasmids to extracranial carotid arteries without interrupting blood flow (38–40). Transfer of genes or ODNs into the vascular wall is localized within the area of insonation by this method, although the approach may produce systemic dissemination of ODNs or plasmids.

With the development of methods for gene/nucleotide delivery to cerebral arteries, functional efficacy of the methods has been tested in normal animals before being applied to disease models. For example, delivery of endothelial nitric oxide synthase (eNOS) gene into the vessel wall has been studied (26,30,41). Overexpression of eNOS, in endothelium or even in adventitia, enhances agonist-induced, nitric oxide (NO)-mediated vasorelaxation.

Currently, genes or nucleotides can be delivered only to some accessible segments of cerebral arteries, as discussed earlier. Further improvements in methods for delivery to cerebral arteries that are currently not accessible will provide more effective and selective targeting of gene/nucleotide delivery. In addition, cells in different layers of the vessel wall are transfected heterogeneously. For example, medial smooth muscle cells usually are not transfected, whereas endothelium and adventitia are the primary sites of gene delivery and expression after adenovirus-mediated gene transfer (27,33,42). Therapeutic effects in the medial layer of the vessel wall, however, may be obtained indirectly by alteration of production of biomolecules that are released from endothelial cells or adventitial cells.

## TARGETS OF CEREBROVASCULAR GENE THERAPY

In general, difficulty in maintaining long-term effects is one of the major limitations of current gene therapy techniques, especially for the treatment of chronic diseases. Nevertheless, a transient therapeutic effect may be suitable for treatment of several cardiovascular disorders, such as prevention of restenosis after angioplasty, and therapeutic angiogenesis for ischemic tissue, as suggested previously (10). In this context, chronic cerebral ischemia with poor collateral circulation and acute brain diseases or insults accompanied by functional disorders of cerebral arteries, such as cerebral vasospasm, are potential targets of gene therapy. So far, prevention of cerebral vasospasm after SAH has been a major focus of research, and therapeutic angiogenesis in the brain has recently become an additional target. Prevention of neointimal formation after endothelial injury has also been studied extensively in carotid arteries.

### *Cerebral Vasospasm After SAH*

Cerebral vasospasm, which is a delayed and sustained constriction of cerebral arteries after SAH, is refractory to prevention by conventional methods, and may cause severe ischemic damage to the brain (43,44). It is a good target for gene therapy because of the unique characteristics of the syndrome: delayed onset of vasospasm allows a period of time for transgene expression or downregulation of expression of a specific gene, and the transiency of the risk of the syndrome will not require prolonged alteration of gene expression. In addition, the perivascular approach via CSF for gene/nucleotide delivery

allows alteration of gene expression in arteries over a wide area at the base of the brain, which are exposed to subarachnoid blood. This approach has been used in reported gene therapy experiments for cerebral vasospasm. For adenovirus-mediated gene transfer, the presence of subarachnoid blood does not attenuate transgene expression in cerebral vessels (45,46).

Although mechanisms that lead to cerebral vasospasm are not fully understood, recent evidence suggests involvement of multiple mechanisms (47,48). Several strategies of gene therapy for vasospasm, including enhancement of vasodilator mechanisms, downregulation of vasoconstrictor signaling, and modification of stress-induced cellular responses, have been tested as summarized in Table 1.

Effects of adenovirus-mediated gene transfer of eNOS have been examined in animal models of SAH. The rationale for this approach is that there is evidence for impairment of endothelium-dependent, NO-induced vasorelaxation after SAH (49). In these studies, however, overexpression of eNOS in cerebral arteries generally failed to prevent vasospasm (50,51), although agonist-induced, NO-mediated vasorelaxation was improved in *ex vivo* organ bath studies (51). Because NOS activity and NO production were elevated after eNOS gene transfer (50), failure to prevent vasospasm may be due to inactivation of NO by oxyhemoglobin (47,52) or decreased cellular levels of cyclic guanosine monophosphate (cGMP) (53,54).

In contrast to the attempts to enhance NO-mediated vasorelaxation after SAH, gene transfer of calcitonin gene-related peptide (CGRP), a vasodilator neuropeptide, prevented vasospasm after SAH (55,56) (Fig. 2A). Gene transfer of CGRP increases the level of cAMP in the basilar artery (28), and this is thought to result in activation of K<sup>+</sup> channels, such as adenosine triphosphate (ATP)-sensitive K<sup>+</sup> channels (K<sub>ATP</sub> channels), producing hyperpolarization of smooth muscle membrane and relaxation of cerebral arteries (57,58). This strategy has a good rationale, because the smooth muscle cell membrane is depolarized, and relaxation in response to activators of K<sub>ATP</sub> channels is preserved or enhanced in cerebral arteries after experimental SAH (59–61).

Several other studies have examined the effects of the downregulation of genes related to smooth muscle contraction. The potent vasoconstrictor endothelin-1 (ET-1) and mitogen-activated protein kinase (MAPK), a key intracellular signaling molecule that regulates contraction of smooth muscle in some vessels, have been implicated in the pathophysiology of cerebral vasospasm after SAH (47,62–64). In order to downregulate the expression of these molecules, an antisense ODN against preproET-1 or MAPK was injected into CSF of animals with experimental SAH (25,65,66). Although this approach was reported to be effective in small animals, little effect was found in larger animals, perhaps because a physical barrier of subarachnoid clot prevented ODNs from entering into cerebral arteries (65).

Modification of stress-induced cellular responses by inhibition of nuclear factor (NF)κB, a key transcription factor in vascular inflammatory responses, or by overexpression of heme oxygenase-1 (HO-1), an inducible isoform of key enzymes for catabolism of heme, has been tested after SAH. The rationale is that inflammatory responses in cerebral arteries are implicated in the pathogenesis of cerebral vasospasm (67–69), and induction of HO-1 is thought to be a protective mechanism in cerebral arteries after SAH (70–72). Both strategies, with the administration of “decoy” ODN for NFκB into CSF or by adenoviral gene transfer of HO-1 to cerebral arteries, were effective in reducing vasospasm after experimental SAH (32,73).

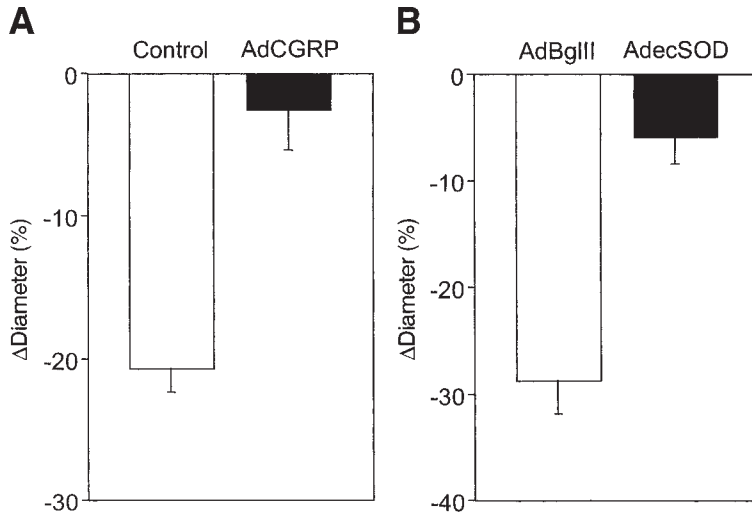
Table 1  
Gene Therapy for Prevention of Vasospasm After Experimental Subarachnoid Hemorrhage (SAH)

<i>Target molecules</i>	<i>Strategy</i>	<i>Gene/nucleotide delivery</i>	<i>Treatment protocol</i>	<i>Reduction of vasospasm</i>	<i>Animals</i>	<i>Refs</i>
eNOS	Gene transfer	Adenovirus	At the time of SAH 24 h before SAH	No Yes	Dog Dog	50 51
preproCGRP	Gene transfer	Adenovirus	30 min after SAH	Yes	Rabbit	55
preproET-1	Antisense	ODN	30 min after SAH 24 h before HI	Yes <sup>a</sup> Yes <sup>b</sup>	Dog Dog Rat	56 65 25
MAPK	Antisense	ODN	At the time of SAH	Yes	Rat	66
NF-κB	Decoy	ODN	At the time of SAH	Yes	Rabbit	32
HO-1	Gene transfer	Adenovirus	At the time of SAH	Yes	Rat	73
ecSOD	Gene transfer	Adenovirus	30 min after SAH	Yes	Rabbit	78

eNOS, endothelial nitric oxide synthase; CGRP, calcitonin gene-related peptide; ET-1, endothelin-1; MAPK, mitogen-activated protein kinase; NF-κB, nuclear factor-κB; HO-1, hemeoxygenase-1; ecSOD, extracellular superoxide dismutase; ODN, oligodeoxynucleotide; HI, hemolytate injection.

<sup>a</sup>In combination with recombinant tissue plasminogen activator.

<sup>b</sup>Hemolytate-induced spasm.



**Fig. 2.** Reduction of vasospasm following subarachnoid hemorrhage (SAH) after gene transfer of calcitonin gene-related peptide (CGRP) or extracellular superoxide dismutase (ecSOD) in rabbits. Adenovirus encoding preproCGRP (AdCGRP) or ecSOD (AdecSOD) was injected into the cisterna magna 30 min after intracisternal injection of autologous blood. The percentage changes in the diameter ( $\Delta$ diameter) of the basilar artery were evaluated by vertebral arteriography performed before and two days after SAH. **(A)** Effects of AdCGRP. Control, animals injected with artificial cerebrospinal fluid or adenovirus encoding  $\beta$ Gal. **(B)** Effects of AdecSOD. AdBgIII, animals injected with adenovirus containing no transgene. Values are mean  $\pm$  SEM.

As a means for enhancement of cellular protection, gene transfer of superoxide dismutase (SOD) has also been tested. Elevated superoxide levels in the subarachnoid space may contribute to cerebral vasospasm (74–76). Superoxide impairs NO-mediated relaxation of cerebral arteries by inactivation of NO, and may trigger oxidative cell damage by production of peroxynitrite and other reactive oxygen species, such as hydroxyl radical. Gene transfer of extracellular SOD (ecSOD), an isoform of SOD that binds to extracellular matrix of vascular tissue, reduced vasospasm (77,78) (Fig. 2B).

Several positive results suggest that prevention of vasospasm after SAH is a promising target for clinical use of the gene therapy technique. Most studies, however, have been performed in SAH models of small animals, in which severity and temporal profile of vasospasm are different from those in humans. Thus, preclinical experiments are needed in larger animals. In addition, mechanisms of development, maintenance, and resolution of cerebral vasospasm are not fully understood yet, so there is no clear target for treatment of vasospasm at present. Further research addressing pathogenesis of vasospasm will greatly benefit the development of effective gene therapy for vasospasm after SAH.

### *Collateral Circulation in the Brain*

Development of collateral circulation in ischemic tissue is an alternative strategy to recanalization of an occluded artery. This strategy, also referred to as “therapeutic angiogenesis,” has been applied in clinical trials on the treatment of myocardial or limb ischemia by gene transfer of angiogenic growth factors, such as vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and basic fibroblast growth factor (bFGF) (*see ref. 79 for review*). In recent studies using a cerebral hypoperfusion model in rats, deliv-

ery of the genes for HGF or VEGF into CSF stimulated angiogenesis in the brain and increased cerebral blood flow (80). Gene transfer of bFGF is also effective in inducing neovascularization in the brain of normal rats (81).

Thus, therapeutic angiogenesis may be applicable to treatment of chronic cerebral ischemia/hypoperfusion produced by disease of extracranial arteries. It is not yet clear, however, whether gene therapy for angiogenesis will be useful for treatment of focal brain ischemia. Neovascularization in the ischemic limb of mice is effectively achieved by intravenous administration of bone marrow-derived endothelial progenitor cells (EPCs) transfected *ex vivo* with the VEGF gene (82). EPC-mediated gene therapy may be applicable to focal cerebral ischemia, because EPCs contribute to neovascularization in the ischemic area of the brain after occlusion of the middle cerebral artery in mice (83).

After accumulation of experience from clinical trials in peripheral circulation, several issues have been raised regarding safety of gene transfer technique for therapeutic angiogenesis (10). For cerebral circulation, it is possible that vascular malformations may develop at the site of angiogenesis, and predispose to brain hemorrhage. In addition, brain edema is a possible complication after gene transfer of VEGF, which increases vascular permeability and produces edema in limbs after gene transfer. Angiogenesis induced by gene transfer of HGF, however, may not produce brain edema (84).

### *Stenotic Lesions of the Carotid Artery*

Restenosis of arteries, which is a major problem after coronary angioplasty and stenting, is a potential target for gene therapy (85). For treatment of stenotic lesions of carotid arteries, carotid endarterectomy and angioplasty with stenting are now widely used. Restenosis of the carotid artery after those procedures may be an appropriate target for gene therapy, although the incidence of restenosis in the carotid artery may not be as high as in coronary and iliofemoral arteries (86–88). Neointima formation after endothelial injury in the carotid arteries of small animals is widely used as an experimental model for restenosis after angioplasty, and gene therapies have been tested for inhibition of neointima formation using this model (Table 2).

The most common approach in early studies for the prevention of neointima formation was direct inhibition of smooth muscle proliferation by inducing cell-cycle arrest. Several strategies, including antisense ODN-based gene targeting of essential components or positive regulators of the cell cycle (89–93), inhibition of the key transcription factor E2F by decoy ODN administration (94), and gene transfer of negative regulators of the cell cycle (95–100) were found to be effective in reducing neointima formation of carotid arteries. Some of those approaches have been applied to patients with coronary artery disease in clinical trials (3,101).

Other studies have demonstrated a reduction of neointima formation by inhibition of smooth muscle proliferation and/or migration in injured carotid arteries through several approaches, such as downregulation of expression of growth factors or a growth factor receptor by antisense strategies (102–104), gene transfer of dominant negative mutants of mitogenic signaling molecules (37,105,106), and applications of decoy ODNs for key transcription factors in smooth muscle proliferation (107,108). Recent studies have suggested efficacy of other approaches, including facilitation of apoptosis of neointimal smooth muscle cells by changing expression of apoptotic or antiapoptotic factors (109,110) and inhibition of migration of medial smooth muscle cells by gene transfer of tissue inhibitors of metalloproteinase (111,112).

Table 2  
Gene Therapy for Carotid Stenosis After Arterial Injury

Category	Target gene/molecules	Strategy	Gene/nucleotide delivery	Refs
Cell cycle	cdc2 kinase, cdk2 kinase, PCNA, cyclin B1, <i>c-myc</i> , <i>c-myc</i> E2F	Antisense	HVJ-L, ODN	89–93
Growth factor	p21 <sup>CIP1</sup> , p27 <sup>KIP1</sup> , GAX, GATA-6, p53, Rb protein	Decoy	HVJ-L	94
Mitogenic signal	bFGF, TGF- $\beta$ 1	Gene transfer	Adenovirus	95–100
	PDGFR $\beta$ -chain	Antisense	Adenovirus, plasmid	102,104
	H-ras, MAPKK, STAT3 (dominant-negative mutants)	Antisense	ODN	103
	AP-1, Egr-1	Gene transfer	Plasmid, adenovirus	37,105,106
Apoptotic signal	Fas-ligand	Decoy	ODN	107,108
	bcl-xL	Gene transfer	Adenovirus	109
	TIMP-1, TIMP-2	Antisense	ODN	110
Metalloproteinase inhibitor		Gene transfer	Adenovirus	111,112
NO-cGMP system	NOS isoforms, PKG catalytic domain	Gene transfer	HVJ-L, adenovirus	115–117,120
PGI <sub>2</sub>	PGI <sub>2</sub> synthase	Gene transfer	Plasmid, HVJ-L	118,119
Antithrombotic	Hirudin, TFPI	Gene transfer	Adenovirus	121,122

PCNA, proliferative cell nuclear antigen; Rb, retinoblastoma; MAPKK, mitogen-activated protein kinase kinase; STAT, signal transducer and activator of transcription; PDGFR, platelet-derived growth factor receptor; AP-1, activator protein-1; Egr-1, early growth response factor-1; bFGF, basic fibroblast growth factor; TGF, transforming growth factor; TIMP, tissue inhibitor of matrix metalloproteinase; NOS, nitric oxide synthase; PKG, protein kinase G; PGI<sub>2</sub>, prostaglandin I<sub>2</sub>; TFPI, tissue factor pathway inhibitor; ODN, oligodeoxynucleotide; HVJ-L, hemagglutinating virus of Japan-liposome complex.

Facilitation of re-endothelialization is an attractive concept for treatment of injured arteries. In rabbit femoral artery, gene transfer of VEGF reduced formation of neointima and the frequency of thrombotic occlusion by accelerating re-endothelialization after endothelial injury (113). Gene transfer of VEGF has been tested for prevention of coronary restenosis in patients undergoing angioplasty (114). Reconstitution of production of endothelium-derived mediators with antiproliferative and antithrombotic properties, such as NO and prostaglandin I<sub>2</sub> (PGI<sub>2</sub>), in the injured vessel wall is an alternative strategy to facilitation of re-endothelialization. Gene transfer of NOS isoforms (115–117) or PGI<sub>2</sub> synthase (118) has been reported to be effective for reducing neointima formation in injured carotid arteries. Overexpression of PGI<sub>2</sub> synthase also has been reported to accelerate endothelial regeneration after balloon injury (119). In accordance with these studies, neointima formation was reduced by gene transfer of the catalytic domain of protein kinase G (120), or antithrombotic molecules, such as hirudin or tissue factor pathway inhibitor (121,122).

Thus, there are many possible choices for gene therapy for carotid restenosis. Results of most studies, however, may not be directly relevant to clinical settings, because of the challenges of intraluminal gene/nucleotide delivery with interruption of carotid blood flow. Several studies, however, suggest feasibility of the perivascular approach (for examples, *see refs. 37,92,103*). A solution for the problem of the intraluminal approach may be provided by recent developments in catheter- or stent-based gene/nucleotide delivery systems, which may enable safe and effective intraluminal approaches for the carotid artery (123–125). Again, experimental models in most studies involve small animals, and long-term outcome of the treatments is unclear because the follow-up period in most studies is only a few weeks. Preclinical studies with an experimental design that is more relevant to the clinical setting are needed.

Prevention of thrombotic events, including both acute occlusion at the lesion site and thromboembolism to distal arterial branches, is also important for patients with carotid atherosclerotic lesions, even if the lesions do not produce hemodynamically significant stenosis. For this purpose, regression or stabilization of plaques is an appropriate target for gene therapy. Several studies have demonstrated feasibility of gene therapy for atherosclerotic lesions in the aorta of mice (126–128).

## SAFETY AND FUTURE DIRECTIONS OF CLINICAL APPLICATIONS

After reasonably successful and promising initial steps toward the establishment of cerebrovascular gene therapy, concerns about safety have emerged as the major obstacle and urgent problem. Concerns about systemic adenoviral toxicity and insertional mutagenesis induced by a retroviral vector have been reinforced after serious adverse effects were reported in patients from clinical trials (6,7). In relation to cerebrovascular gene therapy, systemic toxicity after adenovirus-mediated gene transfer is unlikely because administration of adenoviruses is local. Similarly, because adenoviruses currently are used predominantly, insertional mutagenesis is not an issue. Inflammatory and immune responses caused by adenoviral vectors, however, are problematic even after local administration. For example, inflammatory changes in cerebral arteries after adenoviral gene transfer may exacerbate vasospasm because inflammation is an important characteristic in cerebral vessels after SAH (67–69).

Intense efforts are underway to minimize adverse effects of adenoviral vectors, because adenoviral vectors are a very promising choice of vectors. A simple method is concomi-

tant use of cationic polymer/lipids or calcium phosphate, which enhances transgene expression in cerebral arteries, and thereby allows use of lower titers of adenoviral vectors (129,130). A technology for constructing a new generation of adenoviral vectors also may help to reduce adverse effects (19). Helper-dependent, or “gutless” or “guttled,” adenoviral vectors, which do not contain most genes encoding viral proteins, allow efficient transfection but produce less inflammatory and immune responses than other adenoviral vectors. A recent study demonstrated long-term transgene expression after systemic gene transfer with this vector in mice (127), although dose-dependent acute toxicity has been reported in nonhuman primates (131). A relatively minor challenge is purification, with contamination by the helper virus, which is required for production of the helper-dependent adenovirus. A great challenge, however, is large-scale production of the vector. Improvements in production of the vector may address concerns about large-scale production (132,133).

Tissue-selective targeting of vectors and/or transgene expression is useful for reducing adverse effect and increasing therapeutic effect (134,135). For example, over-expression of VEGF gene can be regulated by a hypoxia-inducible enhancer (136). This method may enable selective angiogenesis in ischemic tissue and prevent unwanted, excessive angiogenesis in normal tissue.

For the safe use of vectors, data regarding the pharmacokinetics of vectors in animals, and ultimately in humans, will be required (137). In addition, even when a beneficial effect is anticipated, adverse effects of specific genes or nucleotides must be clarified. Because cerebrovascular gene therapy targets vascular tissue in close proximity to neurons, evaluation of changes in brain function, including the neuropsychological effects, induced by transgene products or nucleotides will be important.

## CONCLUSION

Cerebrovascular gene therapy is still in its infancy, and many basic studies have yet to be performed in order to fulfill minimum requirements for its clinical use. Some aspects of the fundamental technology for gene therapy have been developed but, in working toward cerebrovascular gene therapy trials, further improvements are needed for production of safe and effective vectors. In addition, continued advances in understanding the biology of diseases and vectors will be useful (8).

It is unlikely that cerebrovascular gene therapy will be used widely in patients in the near future, but we are optimistic that improvement of methods for gene therapy will ultimately result in its clinical use.

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# III

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## REGENERATIVE TISSUES FOR THE DISEASED CARDIOVASCULAR SYSTEM

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# 16

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## Therapeutic Angiogenesis and Vasculogenesis for Tissue Regeneration

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*Paolo Madeddu, MD, FAHA*

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### SUMMARY

Therapeutic angiogenesis/vasculogenesis represents a new approach to treating patients with ischemic disease not curable with conventional treatment. Manipulation of the angiogenesis program and transplantation of progenitor cells is hoped to overcome endogenous liabilities that impede appropriate healing in atherosclerotic or diabetic patients. Viral vectors represent the usual means for delivering curative genes, but new nonviral methods are gaining importance for their safer profile. The number of angiogenic substances suitable for therapeutic purposes is rapidly growing, and combinatory strategies offer distinctive advantages. Combating endothelial death or interfering with vascular destabilization may prevent organ failure. Ex vivo engineered endothelial progenitor cells have been proposed for the treatment of peripheral and myocardial ischemia. The approach eliminates the drawback of immune response against viral vectors and makes repeating the therapeutic procedure in case of injury recurrence feasible. Genetic manipulation of stem cells opens new avenues for regenerative medicine.

**Key Words:** Angiogenesis; vasculogenesis; viral vectors; gene therapy; endothelial.

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## INTRODUCTION

Gene therapy consists of the transfer of nucleic acid sequences to somatic cells for therapeutic purposes. The gene can be directly transferred to the host, or first delivered to cells in culture to make them capable of synthesizing transgene product. Transfected cells can then be introduced into the recipient organism, where they start releasing the therapeutic agent. One of the most promising applications of gene therapy is the promotion of vascular growth. Angiogenesis gene therapy and somatic progenitor cell transplantation have opened new therapeutic possibilities for patients with ischemic disease not curable with conventional treatment. Optimization of the two approaches is now required to increase efficacy and minimize possible side effects. In the near future, it is expected that gene therapy and stem cell transplantation will be used in combination because of the significant technical overlap and reciprocal advantages of the two strategies (1).

## DIFFERENT MODELS OF VASCULAR GROWTH

### *Angiogenesis*

Postnatal neovascularization was originally considered to consist of angiogenesis, i.e., activation of preexisting endothelial cells (ECs) which proliferate, migrate, and sprout *in situ*. Angiogenic sprouting is essential for the development of several physiological and pathological settings, such as endometrial proliferation, postischemic recovery, wound healing, and cancer growth (2). Angiogenesis occurs in stages: (1) vasodilatation and extravasation of plasma proteins that provide the provisional scaffold for migrating ECs; (2) interruption of EC mutual contact and detachment from basement membrane with contribution of extracellular matrix proteinases; (3) EC migration and tube formation; (4) stabilization and remodeling of newly formed vessels into three-dimensional networks; and (5) destabilization and regression of unnecessary microvessels.

Growth factors (GFs) that guide EC proliferation and migration are named direct angiogenic factors. In addition, indirect angiogenic GFs modulate the release of direct factors from cells recruited into sites of angiogenesis. Factors with potential for therapeutic angiogenesis have been classified as belonging to GF families, chemokines, transcription factors, and substances with pleiotropic activity (Table 1).

Under conditions of anemia, hypoxia-inducible transcription factor triggers a coordinated response by inducing expression of endothelial GFs (3). Angiogenesis is also induced by metabolic stimuli, including hypoglycemia and acidosis.

### THE VEGF FAMILY

Vascular endothelial growth factor (VEGF) represents the prototypical angiogenic factor (Fig. 1). By interacting with endothelial tyrosine kinase VEGF receptors VEGFR-1 (flt-1) and VEGFR-2 (flk-1/KDR), VEGF-A regulates the progression phase of angiogenesis and induces ECs to proliferate, migrate, and survive (4). VEGF-B exists as two protein isoforms, VEGF-B<sub>167</sub> and VEGF-B<sub>186</sub>, resulting from alternatively spliced mRNA. VEGF-B specifically binds to VEGFR-1. However, it also forms heterodimers with VEGF-A, a property that influences receptor specificity and final biologic effects. Recent studies indicate that VEGF-B promotes angiogenesis in association with an activation of Akt and eNOS-related pathways (5). VEGF-C, with related receptors VEGFR-2 and VEGFR-3 (flt-4), represents an apparently redundant pathway for postnatal angiogenesis. VEGF-C was shown to stimulate nitric oxide (NO) release from ECs and

Table 1  
Factors With Angiogenic Properties

<i>Growth factors</i>	<i>Chemokines</i>	<i>Transcription factors</i>	<i>Pleiotropic substances</i>
VEGF Family	MCP-1	HIF	Tissue kallikrein
FGF Family		EGR	PAR-activators
Angiopoietins		Prox	Thrombin
HGF, PDGF-BB		Cyr61	
IGF-1, IGF-2			Secreted frizzled-related protein
NGF			Nitric oxide

VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; PDGF, platelet-derived growth factor; IGF, insuline-like growth factor; NGF, nerve growth factor; MCP-1, monocyte chemoattractant protein-1; HIF, hypoxia-inducible factor; EGR, early growth response gene; PAK, protease-activated receptor.

to induce neovascularization in a rabbit model of hind limb ischemia (6). Evidence also indicates a role for VEGF-C in pathological angiogenesis and lympho-angiogenesis (7).

#### THE ANGIOPOIETINS FAMILY

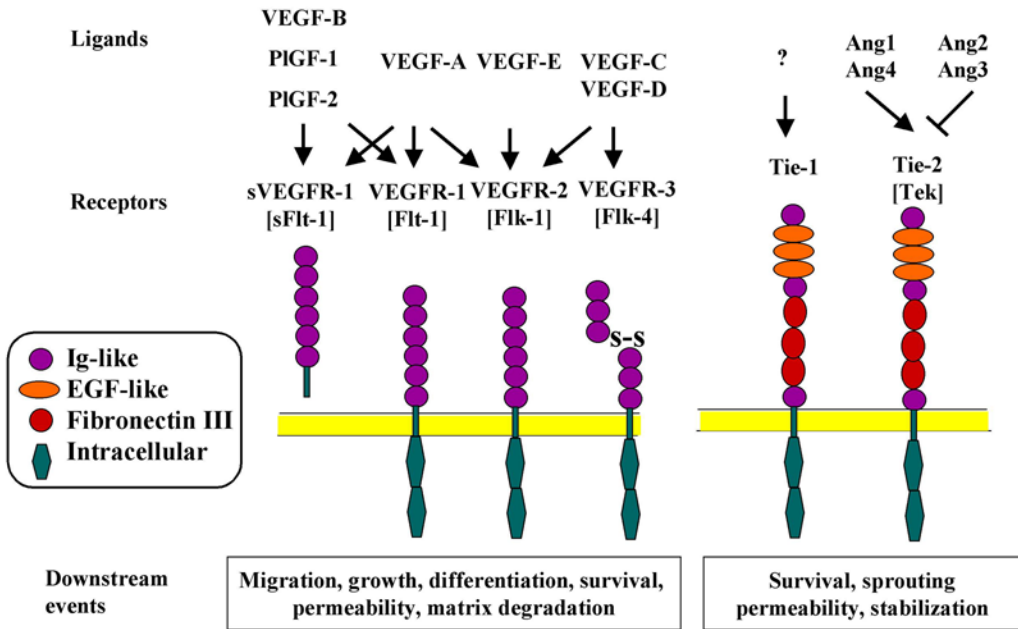
Angiopoietin-1 (Ang-1), which acts through the Tie-2 receptor, is essential for normal embryonic development, whereas in the adults, it decreases vascular permeability and stabilizes networks initiated by VEGF, presumably by stimulating an interaction between ECs and pericytes (Fig. 1) (8,9). Ang-2 is highly expressed at sites of normal and pathological vascular remodeling. It contributes to stabilizing the shape of native vessels, rendering a quiescent capillary responsive to VEGF-A. However, in the absence of the appropriate stimulus for vessel growth, expression of Ang-2 in endothelium is associated with vessel regression (10).

#### THE FGF FAMILY

Members of fibroblast growth factor (FGF) family promote the recruitment of mesenchymal cells to vessel walls, an essential mechanism for muscularization of nascent capillaries (11,12). FGF-2 activates the extracellular signal-regulated kinase 1 and 2 (ERK 1/2) through its specific receptors. It has been implicated in protective pathways that increase cell survival, promote neovascularization, and elicit cardioprotection. However, FGFs also contribute to cancer angiogenesis.

#### PLEIOTROPIC AGENTS

The number of newly discovered angiogenic substances is rapidly increasing. Recently, a role for the kallikrein-kinin system in angiogenesis has been proposed by our group (Fig. 2) (13,14). Knockouts for kallikrein and kinin receptors are viable, thus suggesting that the system is not essential to embryo vasculogenesis. In adulthood, kinin B<sub>1</sub> receptor and tissue kallikrein gene expression is upregulated under conditions of myocardial or limb ischemia (13,15). The functional importance of these expressional changes is documented by the fact that native angiogenic response to ischemia is blunted by chronic B<sub>1</sub> receptor antagonism or genetic deletion of the same receptor (16). Furthermore, disruption of B<sub>2</sub> receptor gene results in myocardial capillary rarefaction and ischemia with aging (17).



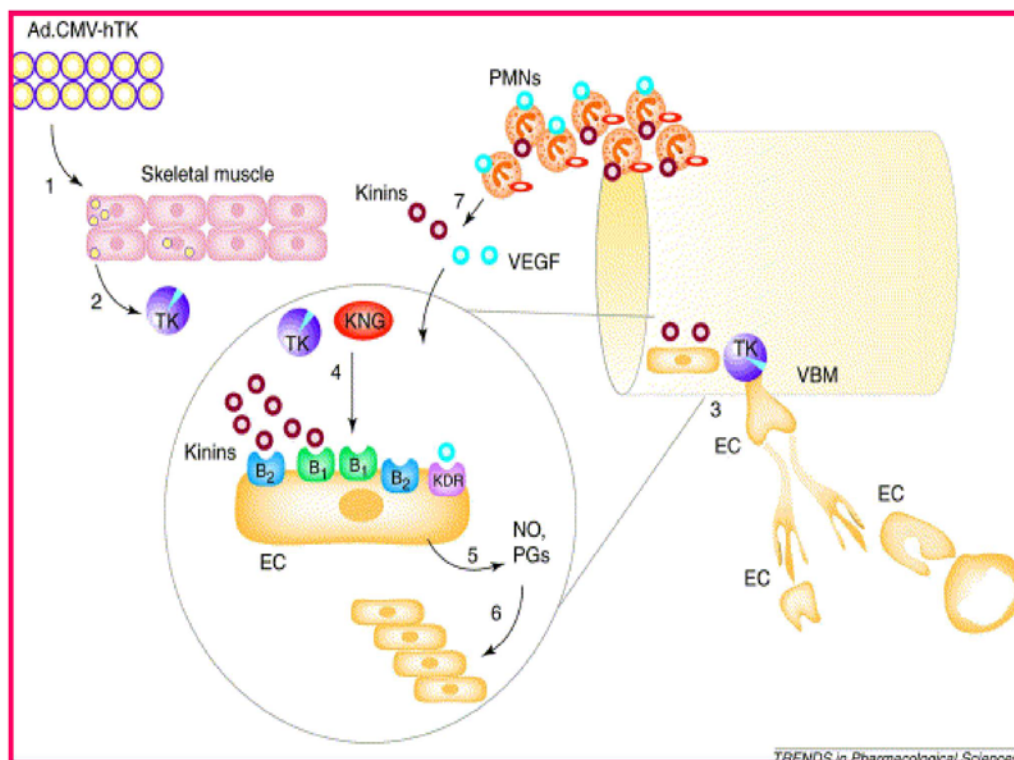
**Fig. 1.** The vascular endothelial growth factor (VEGF) and angiopoietin families. VEGF and angiopoietins cooperate in the generation of new vessels and their rearrangement. VEGF interacts with endothelial tyrosine kinase VEGF receptors VEGFR-1 (flt-1) and VEGFR-2 (flk-1/KDR); VEGF-A regulates the progression phase of angiogenesis and induces endothelial cells to proliferate, migrate, and survive. Other isoforms of VEGF are displayed together with their receptors. A soluble form of VEGFR-1 (s-flt-1) is present in circulation and may form complexes with VEGFs, thus inhibiting their biological functions. Angiopoietins activate Tie receptors and interact with VEGF for vascular stabilization. Placental growth factor (PIGF) belongs to VEGF family and exerts its function through bimolting of VEGF-1.

**NEUROTROPHIC CONTROL OF ANGIOGENESIS**

The neurotrophin nerve growth factor (NGF), known to regulate neuronal survival and differentiation, acts as a stimulator of angiogenesis and arteriogenesis (18). Thus, chemical signals derived from nerves may drive vascular growth. In addition, NGF high-affinity tyrosine kinase receptor (trkA) and low-affinity p75 receptor are present on ECs and vascular smooth muscle cells (VSMC), suggesting the existence of paracrine/autocrine loops of the polypeptide on these cells. NGF exerts direct mitogenic and prosurvival effects on ECs via trkA phosphorylation and subsequent activation of ERK1/2 and VEGF/phosphatidy1-3' kinase (PI3K)/Akt-B/NO pathways (18). Instead the functions of p75 remain undefined, although recent studies support a dual role for p75 acting as co-receptor modulating NGF-mediated trkA activity, as well as promoter of signaling cascades that result in the induction of apoptosis. Thus, relative expression of NGF subtype receptors on cellular surface can provide a bifunctional switch for survival or death decisions (19).

**SEMAPHORINS' GUIDANCE OF NEOANGIOGENESIS**

The motility and morphogenesis of ECs is controlled by spatio-temporally regulated activation of integrin adhesion receptors, and integrin activation is stimulated by major



**Fig. 2.** Mechanisms mediating kallikrein-induced angiogenesis. Tissue kallikrein gene was delivered to skeletal muscle via a viral vector. Transduced protein is released from myocytes and generate kinins. Binding of kinins with receptors on endothelial cells stimulates nitric oxide and prostacyclin release. Kinins also stimulate the recruitment of inflammatory cells that are rich of growth factors. In addition, kallikrein induces the activation of matrix proteinases. Recent studies indicate that kallikrein acts through the intracellular kinase Akt, by a mechanism independent of vascular endothelial growth factor. PMNs, polymorphonuclear leukocytes; VEGF, vascular endothelial growth factor; KNG, kininogen; EC, endothelial cell; VBM, vinblastine, methotrexate, and bleomycin; NO, nitric oxide; PGs, prostaglandins (Adapted from ref. 19a.)

determinants of vascular remodeling. During vascular development and experimental angiogenesis, ECs generate autocrine chemorepulsive signals of class 3 semaphorins (SEMA3 proteins) that localize at nascent adhesive sites in spreading endothelial cells. Disrupting endogenous SEMA3 function in ECs stimulates integrin-mediated adhesion and migration to extracellular matrices, whereas exogenous SEMA3 proteins antagonize integrin activation. Expression of dominant negative SEMA3 receptors in chick embryo ECs locks integrins in an active conformation, and severely impairs vascular remodeling. *Sema3a*-null mice show vascular defects as well. Thus, during angiogenesis, endothelial SEMA3 proteins endow the vascular system with the plasticity required for its reshaping by controlling integrin function (20).

### Vasculogenesis

It was principal merit of Asahara and Isner having documented that vasculogenesis, which is *de novo* vessel formation by bone marrow (BM)-derived endothelial progenitor

cells (EPC), plays an important role in postnatal neovascularization triggered by ischemia, wounding, or cancer.

EPCs have been identified not only in bone marrow but also in peripheral blood (21,22). These cells are rapidly mobilized from BM into the circulation from where they colonize ischemic areas and differentiate into mature ECs. Similarly, EPCs are mobilized in areas of vascular trauma or acute myocardial infarction (23,24). The increase in circulating EPCs was positively correlated to increased plasma VEGF levels in these patients (24).

#### MODULATORS OF VASCULOGENESIS

The molecular events regulating vasculogenesis are largely unknown. Mobilization and recruitment of such elements by VEGF is supported by several findings. VEGFR-1 and VEGFR-2 are expressed on EPCs (25). Furthermore, EPCs isolated from circulation are capable of secreting VEGF in liquid culture (26). After VEGF administration in mice, EPCs increase in the circulation- and display-enhanced proliferative and migratory activity (27). Likewise, patients given *VEGF* gene transfer for the treatment of peripheral ischemia show a significant increase in circulating EPCs, thus suggesting that VEGF overexpression can mobilize EPCs. In addition to the direct effects on EPC mobilization, VEGF can also induce the release of hematopoietic GFs, such as granulocyte-macrophage colony-stimulating factor (28,29). Modulation of EPC kinetics has been similarly observed in response to granulocyte colony-stimulating factor and stem cell factor (SCF, sKitL) (29). Matrix metalloproteinase-9 appears to play an essential role for EPC recruitment from the BM, an effect that involves SCF release (30).

The same factors responsible for mobilization may also be implicated in EPC migration and incorporation. For instance, increased statement of VEGF is paralleled by EPC recruitment into sites of vascular injury or exogenous VEGF delivery. In accordance, EPC homing is guided by VEGF and stromal cell-derived factor-1 (SDF-1) (31). The latter binds to the chemokine receptor CXCR-4, which is highly expressed on EPCs (32).

#### *Arteriogenesis*

Arteries provide the bulk flow to tissue; thus, interconnection of the arterial system is necessary to give relief to ischemic organs. The mechanisms of angiogenesis and arteriogenesis differ significantly. Arteriogenesis is driven by shear stress which stimulates EC to send chemoattractant signals to monocytes (33). These cells produce GFs and proteinases, which promote VSMC proliferation. Members of FGF family and platelet-derived growth factor (PDGF)-BB are involved in arteriogenesis, whereas VEGF seems to stimulate capillary growth more efficiently. The identification of substances regulating collateralization has great relevance from a therapeutic viewpoint. Substantial differences exist regarding the rate and characteristics of microvascular growth among species. For instance, reparative angiogenesis is very fast in rats compared with mice. In the rabbit, arteriogenesis is more efficient than in mice and rats. All these differences should be taken into account when attempting to extrapolate therapeutic efficacy of an angiogenic substance from preclinical models to clinical trials.

#### *Vascular Regression*

Vessel destabilization is a natural process that intervenes to finish the angiogenic process once perfusion matches the metabolic request. In general, there is a perfect balance between the number of capillaries and cells. In skeletal and myocardial muscle this ratio is equal to 1. In addition, there is an optimal distance for oxygen and nutrient

diffusion from capillary to cells. When too many vessels are generated, compensatory mechanisms intervene to switch off the angiogenic program. There are, however, different ways by which the angiogenic process is terminated. In healthy animals, neoangiogenesis generally results in a well-organized and persistent vascular network. In contrast, under conditions like diabetes, proliferating ECs are committed to premature death by apoptosis. This generates a vicious cycle that leads to persistent hypoxia, cellular death, and eventually organ failure (34,35).

Thrombospondins inhibit angiogenesis through direct effects on ECs and indirect effects on GF mobilization or activation. Ang-2 acts as a destabilizer at low levels of VEGF expression. Inhibitory PAS domain protein, C-reactive protein, chemokines binding CXCR3, VEGF soluble receptors Flt-1 and Tie-2, and proteinase products (such as fragments of kininogen and angiotensinogen) inhibit angiogenesis and contribute to vessel regression (36).

### DISEASE-RELATED IMPAIRMENT OF ANGIOGENESIS AND VASCULOGENESIS

Under disease conditions, impaired neovascularization results in part from diminished vascular GF production. However, endogenous expression of cytokines is not the only factor responsible for the deficit. In fact, primary dysfunction of ECs may configure a picture that we have defined as endotheliopathy (Fig. 3) (36). For instance, diabetic or hypercholesterolemic animals—like patients affected by similar diseases—exhibit alterations in EC proliferation and viability (37,38). Excessive apoptotic endothelial and muscular cell loss progressively occurs in the hind limb of diabetic animals, and rapid acceleration intervenes at occasion of arterial obstruction (35). Although the cellular dysfunction does not necessarily preclude a favorable response to cytokine replacement therapy, the extent of hemodynamic recovery does not match that of control healthy animals; thus suggesting a limitation imposed by a diminished responsiveness of EPCs/ECs.

Aging, another condition associated to impaired neovascularization, might also lead to dysfunctional EPCs and defective vasculogenesis (39,40). Consistently, EPCs from older patients with clinical ischemia have significantly less therapeutic efficiency in rescuing ischemic hind limb of mice compared with those from younger ischemic patients (41).

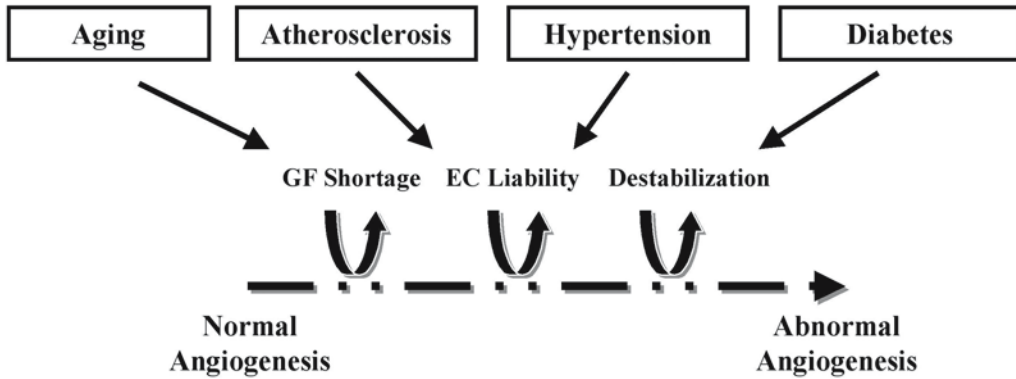
Recently, Vasa et al. have evaluated EPC kinetics and their relationship to clinical disorders. They showed that the number and migratory activity of circulating EPCs inversely correlates with risk factors for coronary artery disease, such as smoking, family history, and hypertension (42). Hill et al. reached the conclusion that circulating EPCs may represent a surrogate biological marker for assessing cardiovascular risk (43). In addition, EPC and monocyte kinetics might provide indications on the therapeutic efficacy of transplantation approach (44).

### THERAPEUTIC ANGIOGENESIS/VASCULOGENESIS: PROMISES AND LIMITATIONS

This novel therapeutic approach is intended to overcome the limits in GF production and endothelial cell dysfunction inherent to aging and degenerative disease.

#### *Delivery Techniques*

For angiogenesis gene therapy to be successful, a curative gene must be delivered to the right target without endangering distant tissues where angiogenesis could be delete-



**Fig. 3.** Aging, atherosclerosis, hypertension, and diabetes alter the function of endothelial cells and hamper the action of growth factors. Excessive activation of apoptosis leads to progressive microvascular rarefaction in diabetic muscle and myocardium.

rious. Delivery systems are mainly based on viral and nonviral vectors. There are various barriers that impede an easy entering of exogenous genetic material into cells (Fig. 4). Genetic material has to be taken up using receptor-mediated mechanisms or endocytosis, followed by endosomal release and nuclear import. Integration into host genome is required for persistent transgene expression.

#### PLASMIDS

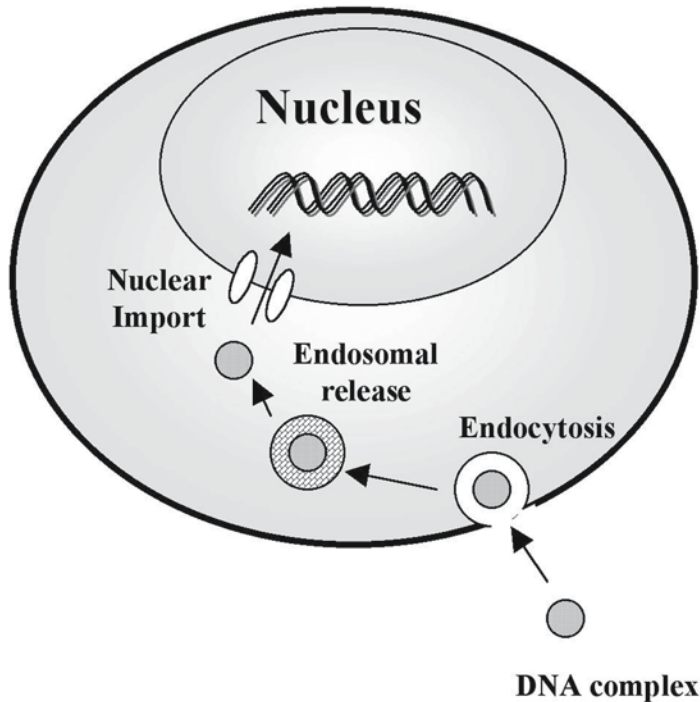
Naked plasmid DNA is taken up by cells with low efficiency. However, muscle tissue represents an exception, because relatively high expression levels have been reported after plasmid injection. Carrier molecules were used to increase plasmid-based gene transfer efficiency, including liposomes or polymer complexes. Plasmids are expressed for short periods, but have the advantage of easier manufacturing and safer profile compared with viral vectors.

#### ADENOVIRAL VECTORS

Recombinant viruses are widely used for gene delivery. Viruses have developed efficient mechanisms to overcome the endosomal barrier. Viral protein often contains membrane-active domains mediating the delivery of viral genome to the cytoplasm after activation in the endosome.

Adenoviral vectors (AV) have been widely used for gene therapy as a result of their ability to transduce nondividing cells very efficiently. They are made replication deficient by producing point mutations, deletions, insertions, and combinations directed toward a specific AV gene or genes, such as the E1 gene. AV vectors can be amplified and produced in high titres ( $10^{11}$ – $10^{13}$  virus particle/mL<sup>-1</sup>) and accommodate transgene cassette up to 10 kb. Further, they allow relatively transient gene expression, a positive prerogative in reparative angiogenesis. AV are also utilized to achieve transient genetic engineering of stem cells.

Most currently used AV are based on serotype 2 and serotype 5. A high prevalence of antibodies against these serotypes is observed in humans, whereas the prevalence of antibodies against serotype 35 is low. The capsid protein is the major target for neutralizing antibodies. Immunogenicity precludes repetition of gene transfer in case of disease recurrence. Therefore, strong efforts have been made to overcome immune reactions.



**Fig. 4.** Genetic material has to pass several obstacles before being incorporated into cells. Generally, exogenous DNA cannot enter the cell, but viruses exploit endocytosis for incorporation.

Construction of chimeric adenovirus vectors may offer definite advantages with respect to immunogenicity, thereby allowing more durable transgene expression. Chimeric adenoviral vectors, Ad5/H3, generated by replacing the *Ad5* hexon gene with the hexon gene of Ad3, were not neutralized by antibodies in mice previously injected with Ad5 adenoviral vectors (45).

#### ADENO-ASSOCIATED VIRAL VECTORS

Adeno-associated virus (AAV) is a small single-strand DNA parvovirus that is defective, nonpathogenic, and largely diffused in the general population. More than 90% of the parental viral genome is deleted in AAV-based vectors. Consequently, no viral protein is expressed from AAV vectors in transduced cells. Recent advances enhanced the production capability of high-titer stocks of AAV. Accordingly, vectors that express different genes can be mixed before transduction in order to obtain the simultaneous statement of two or more different proteins in the same tissue. AAV has been shown to infect primate hematopoietic progenitor cells with reasonable efficiency; but its use involves coinfection with a helper virus, such as adenovirus or herpes simplex virus. Use of recombinant AAV will require the development of optimized cotransfection and helper plasmid strategies for productive infection. Finally, AAV has a broad host range, but displays an exquisite tropism for nervous and muscular tissue. The efficiency of AAV transduction in skeletal and cardiac muscle cells, as well as in smooth muscle cells, is very high, and transgene statement persists for long periods of time. It is possible to insert regulatory motifs in coding sequence so as to switch off transgene expression whenever



necessary. All these properties make AAV vectors valuable tools for delivery of single factors or of their combination in therapeutic angiogenesis and vasculogenesis.

### RETROVIRAL VECTORS

Retroviral vectors (RV), such as the murine leukemia virus, can accept up to 8 kb of exogenous DNA, and require cell division in order to integrate in their genome. Therefore, most *ex vivo* retroviral gene transfer protocols include the addition of a variety of stimulatory cytokines to induce cell cycling before sequential addition of vector-containing supernatants. Vectors derived from lentivirus (LV) offer major advantages over other RV, because they do not depend on the proliferation of target cells for 'stable' transduction and induce very little immune response. These two features, together with the relative ease of production, make LV vectors an ideal tool for the design of experimental models involving gene transfer into progenitor populations. Recently, LV vectors have been used for stable expression of marker genes in hematopoietic progenitors, thus opening new perspectives to stem cell gene transfer studies. For instance, ECPs harvested from peripheral blood can be engineered to secrete proangiogenic substances and then reintroduced into the organism. However, concerns have been raised that LV-mediated transgene integration into the host genome may induce gene mutations or unregulated transgene expression leading to generation of vascular tumours in the implantation site. Furthermore, the development of leukemia in two children several months after retroviral gene transfer for severe combined immunodeficiency and the demonstration of the causal role of insertional oncogenesis in this process has shed serious doubts on the safety of randomly integrating vectors (46).

Two strategies may avoid insertional oncogenesis: (1) the development of vectors with site-specific integration; and (2) the development of episomal replicative vectors. Most currently used episomal replicative systems are based on the Epstein–Barr virus origin of replication. However, these systems require the use of a viral transactivator (Epstein–Barr nuclear antigen-1). Because the Epstein–Barr virus-based system may be associated with oncogenesis, episomal replicative systems functioning independent of a viral transactivator must be considered in the future.

### NONVIRAL VECTORS

The negative side effects of viral vectors might be prevented by delivery of transgene via synthetic molecules. Cationic lipids and polycationic molecules bind to DNA by electrostatic interactions, leading to hydrophobic collapse with formation of nano-particles, which are resistant to DNase. Masking positive charges to reduce interactions with blood components and proteins can be accomplished with hydrophilic polymers, such as polyethylene glycol. Transferrin has been used for targeting specific receptors which are expressed by plasma membranes. Fab-antibodies for cell surface markers also enabled efficient gene delivery. Synthetic viral proteins have been successfully incorporated in nonviral vectors to enhance intracellular delivery of transferred DNA, including influenza-derived peptides. Nonviral vectors are nonefficient in passing nuclear membrane. Incorporation of peptides containing nuclear localization signals can help overcome this bottleneck.

### TRANSCRIPTION FACTORS

Engineered transcription factors present an advantage over conventional gene transfer, which relies on expression of an exogenous cDNA. Engineered factors can activate or

repress endogenous genes in the appropriate dosage. Rebar et al. documented that zinc-finger transcription factor induces expression of native VEGF isoforms, apparently leading to the formation of a well organized neovascularization instrumental to wound healing (47).

### *Dosage Effects*

At variance with traditional drugs, the effective dosage of gene therapy is hard to predict because it depends on infectivity of the viral vector, the half-life of the angiogenic molecule and its ability to diffuse to adjacent tissue, a peculiarity of those transgenes carrying a secretory signal sequence. In relation to the last assumption, it should be noted however that even angiogenic GF lacking a signal sequence might be released as a result of lysis of infected cells or by a nonclassic pathway.

The localization of transgene product can be monitored in preclinical models of angiogenesis by different techniques, including immunohistochemistry (Fig. 5A). More problematic is tracing transgene expression in patients. In addition, a few studies have addressed the relation between infecting dosage and biological effects (Fig. 5B). We found that low-dosage gene therapy can be successfully applied to stimulate angiogenesis, thus improving the therapeutic/risk index of the strategy. In addition, incremental increases of recombinant angiogenic protein did not necessarily produce additional effects, possibly as a result of the saturation of downstream mechanisms or activation of contraregulatory factors (Fig. 6).

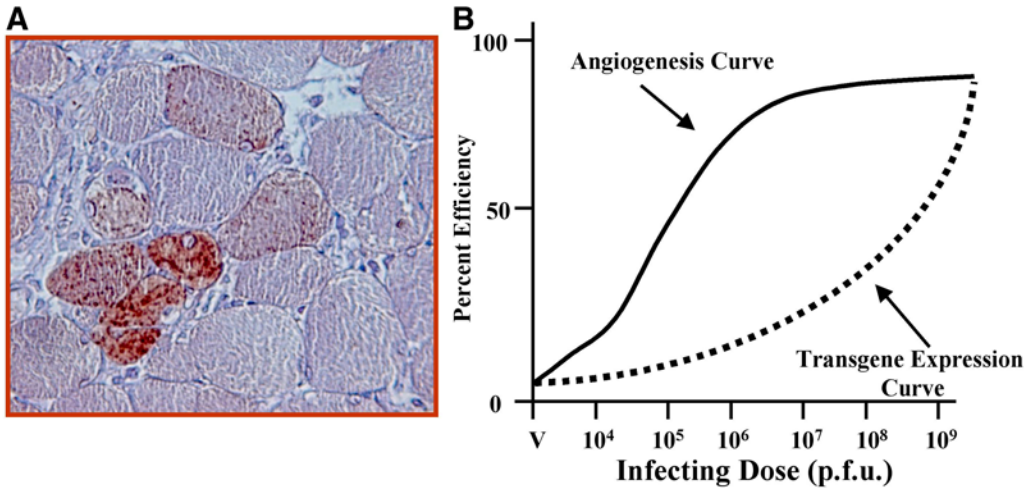
Biological effects of angiogenesis gene therapy are highly dependent on the modality and volume of injection. Inverse transfection efficiency relative to body weight was evidenced by cumulative observations in different species. Mice displayed 60-fold, and rats 50-fold, increased capacity to be infected as compared with pigs or humans. This is because of greater tissue diffusion of gene transfer vectors and smaller volume of rodent skeletal and myocardial muscle. Tissue damage occurs more easily during manipulation of small animals, which may further facilitate transduction efficiency.

### *Gene Targeting to Specific Vascular Sites*

The endothelium once was considered an homogeneous inert population of elements separating circulation from surrounding tissue. Established evidence indicates instead that functional and structural diversity of ECs is the result of molecular differences among EC populations. Recently, organ-specific endothelial antigens have been characterized using *in vivo* phage display, a technique in which peptide libraries, expressed on the surface of bacteriophages, bind to EC surface molecules. *In vivo* phage display creates a map of addresses that can be used for targeting therapeutic agents to tissues affected by vascular disease or cancer. This strategy might be applied to enhance the specificity of therapeutic angiogenesis.

### *Angiogenesis Gene Therapy*

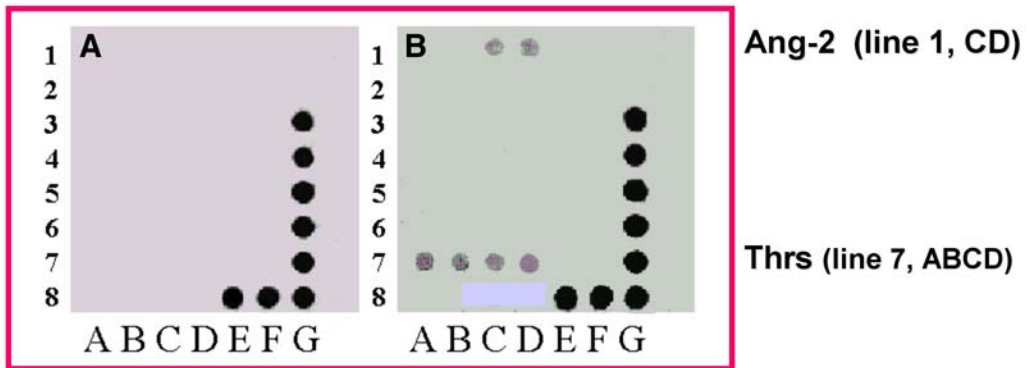
This approach is based on the concept that supplementation with GFs would overcome the endogenous deficit of cytokines and result in more robust angiogenic response (48). Potentiation of microcirculation by therapeutic angiogenesis has been applied in models of myocardial and peripheral ischemia and subsequently exploited for the treatment of wound-healing and peripheral neuropathy. Following successful application in animal models, these concepts have been transferred from the bench to the bedside. However, the results of first controlled clinical trials using VEGF<sub>165</sub> or FGF-2 in patients with ischemic heart disease did not result in the level of efficacy for which researchers had hoped. A list of phase 2 and 3 clinical trials is provided in Table 2.



**Fig. 5.** Immunohistochemical localization of transgenic kallikrein in skeletal myofibers after gene delivery into mouse adductor muscle (A). Dosage-related effect of gene transfer on recombinant protein expression and number of newly formed vessels (B). The curves are markedly different, with recombinant protein increasing exponentially with infecting dosage, whereas vessel growth reaches a plateau at relatively moderate infecting dosage.

The reasons for this partial success are matter of discussion. Preclinical gene therapy protocols are generally performed in otherwise healthy animals, which hardly reflects the condition seen under pathologic conditions in humans. Furthermore, animal studies are carried out without consensus statements on surgical methods to determine ischemia. For limb ischemia, the femoral artery is dissected free from its proximal origin to the bifurcation into the saphenous and popliteal arteries, closed by electro-coagulation or silk ligatures, and excised. More drastic lesions, including femoral vein and nerve, or milder interventions have been reported. For instance, electro-coagulation of the upper part of femoral artery is recommended to avoid excessive sufferance in nonimmuno-competent or otherwise non-healing animals. It is obvious that none of these methods reflects the natural event that, in humans, leads to thrombosis and ischemia. Advanced atherosclerotic lesions can growth sufficiently large to occlude the lumen of a vessel. However, the majority of clinical syndromes, including ischemic stroke and myocardial infarction, predominantly arise from rupture or erosion of a preexisting atherosclerotic plaque and consequent thrombus formation. Furthermore, no animal model really mimics the transient ischemia that occurs in patients with angina or recurrent claudication.

In the majority of clinical trials, one angiogenic substance has been administered. Given the complexity of the angiogenesis program, it appears rather naïve thinking that administration of a single angiogenic molecule would be sufficient to generate a well-tempered and durable neovascularization. Furthermore, unregulated vascular GF overexpression leads to functionally inefficient neovascularization. To overcome the above limitations, combinatory approaches and expression-regulated gene transfer have been proposed. However, because relatively few titration studies have been performed so far, little is known regarding the appropriate dosage, the duration of treatment, and the schedule of combinations. An empirical approach ignoring these aspects is likely to produce a series of hazardous side effects in critical patients, thus raising public hostility and halting research in vascular medicine.



**Fig. 6.** Microarray of angiogenesis gene expression in muscles injected with kallikrein (**B**) or reporter gene luciferase (**A**). Angiogenesis inducer kallikrein stimulates the induction of thrombospondins which are regarded as angiogenesis terminators.

Another unsolved problem regards the phenotypic variability of different vascular beds in terms of responsiveness to angiogenic and antiapoptotic stimuli and the impact of disease states on this variability. This would imply the use of different therapeutic factors to stimulate collateral growth depending on the specific tissue (for instance, heart vs limb) and disease (for instance, atherosclerosis vs diabetic microangiopathy).

### *Therapeutic Vasculogenesis*

Recent evidence suggests that EPCs may provide an opportunity for therapeutic interventions of regenerative medicine. Indeed, transplantation of exogenous EPCs, isolated from adult peripheral blood, cord blood, or bone marrow, has shown great promise as a potential means to combat cardiovascular disease (49–52). In animal models of ischemia, transplanted EPCs augmented reparative neovascularization either through differentiation into mature ECs, or indirectly through paracrine stimulation of resident EC proliferation. Furthermore, EPCs interfere with ischemia-induced cardiomyocyte apoptosis, thus preventing inappropriate cardiac remodeling and failure (53).

Preliminary evidence suggests that the strategy may have therapeutic utility for the treatment of ischemic disease in humans (54). However, many issues must be settled before the EPC transplantation can be extensively and safely applied to patients, including establishing the source, optimal dosage, and cell subfraction most suitable for therapeutic purpose.

EPCs are generally enriched from circulating mononuclear cells based on adherence on fibronectin and labeling with acetylated low-density lipoprotein (LDL) and ulexlectin (55). This definition is now questioned because cells that coexpress specific endothelial and stem/progenitor markers, such as CD34, VEGFR-2/KDR, AC133, and VE-cadherin, are extremely rare in peripheral blood. Accordingly, the term EPCs should be more appropriately attributed to a purified precursor cell population. Because 10% of ECs in the neovascularization are estimated to derive from differentiation of circulating EPC (56), angioblasts might be more abundant in the blood than previously thought (57). Accordingly, angioblast-like properties have been attributed to CD34<sup>+</sup> CD14<sup>+</sup> cells, showing the capacity to differentiate into macrophages, dendritic cells, or ECs depending on environmental cues (57). Alternatively, a small subset of circulating stem cells,

Table 2  
Phase II and Phase III Angiogenesis Trials

<i>Trial</i>	<i>Therapeutic agent</i>	<i>Disease target</i>	<i>Endpoint</i>	<i>n</i>	<i>Results</i>
VIVA	Rec. VEGF prot.	CHD	ETT	178	Negative
FIRST	Rec. FGF-2 prot.	CHD	ETT	337	Negative
TRAFFIC	Rec. FGF-2 prot	PAOD	ETT	190	Positive
GM-CSF	Rec. GM-CSF prot.	CHD	Coll	21	Positive
AGENT	Ad.-FGF-4	CHD	Flow		
VEGF PVDT	Ad.-VEGF <sub>165</sub>	PAOD	ETT	79	Positive
KAT	Ad.-VEGF <sub>165</sub>	CHD	Angiogr	54	Positive
			ST-segm	103	Positive for
			Perfusion		plasmid/liposome
REVASC	Ad.-VEGF <sub>121</sub>	CHD		67	
RAVE	Ad.-VEGF <sub>121</sub>	PAOD			Negative

CHD, coronary heart disease; PAOD, peripheral artery occlusive disease; ETT, exercise tolerance test; GM-CSF, granulocyte-macrophage colony-stimulating factor; *n*, number of patients.

endowed of self-renewal capacity and regenerative potential, could be responsible for the high incorporation and differentiation rate.

However, shortage of EPCs in peripheral circulation, together with disease-related dysfunction, still limits extensive application of primary EPC transplant (58). Another problem regards the substantial loss of functional EPCs in the first few hours after transplantation. The therapeutic implications of this phenomenon probably have been underestimated, and relevance of apoptosis in jeopardizing vasculogenesis still awaits experimental and clinical documentation.

Certain technical improvements could help to overcome the problems delineated above. They include (1) local instead of systemic delivery of EPCs, (2) chemokine supplementation to promote BM-derived EPC mobilization, (3) enrichment procedures or culture-expansion of EPCs, (4) enhancement of EPC function by gene transduction, i.e., gene-modified EPC therapy, and (5) use of selected subpopulation of EPC endowed with capacity of stimulating vasculogenesis and myogenesis (59).

### *Gene-Modified EPC Therapy*

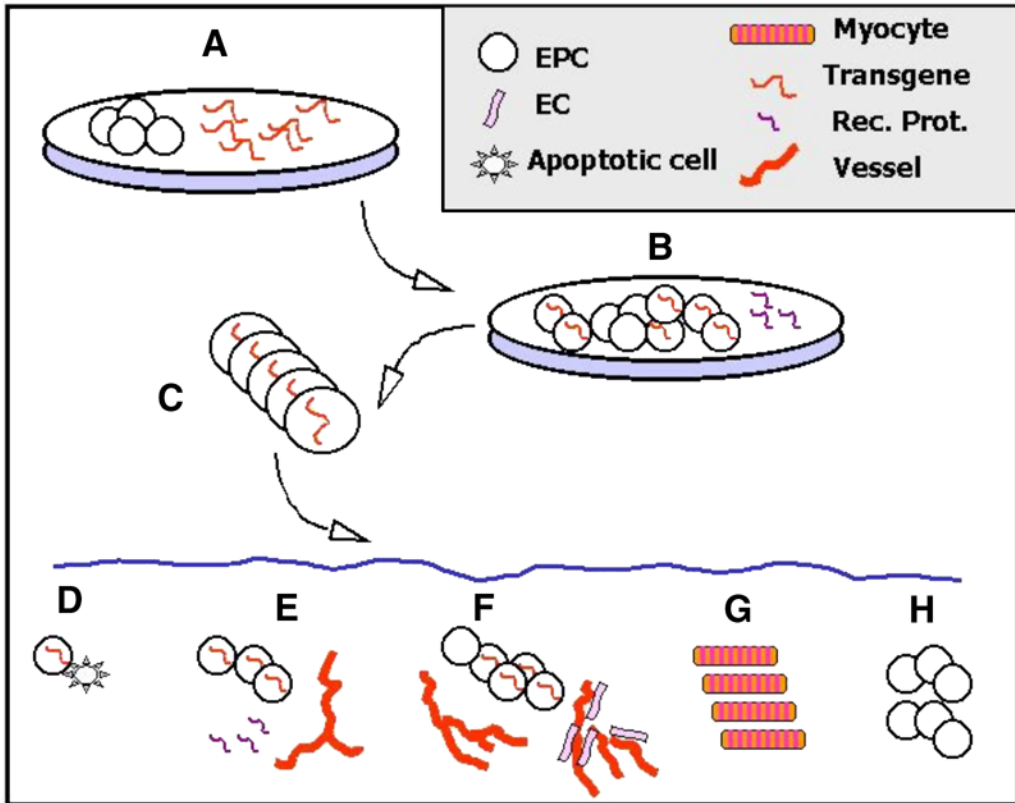
Advances in our ability to genetically manipulate cells *ex vivo* has provided the technological platform to implement EPC biology and circumvent the potential hazard of direct gene transfer. Furthermore, the approach eliminates the drawback of immune response against viral vectors and makes feasible repeating the therapeutic procedure in case of injury recurrence. Another advantage is represented by the possibility of using EPCs as delivery system for curative substances to target organs. Figure 7 shows the major features of *ex vivo* gene therapy.

#### **THERAPEUTIC APPLICATIONS OF TRANSDUCED EPCs**

Transduction of holoclone-generating epidermal cells with RV has led to the first clinical trial of gene therapy for the genetically-determined skin disease epidermolysis bullosa (60). Genetic modification of EPCs to overexpress angiogenic GFs, to enhance signaling pathways implicated in the angiogenic response, and to potentiate biological activity and viability of EPCs is still in its beginning phase, but some therapeutic results have been already reported.

Iwaguro et al. have recently shown that gene-modified EPCs can rescue impaired neovascularization in an animal model of limb ischemia (58). Transplantation of heterologous EPCs transduced with adenovirus-encoding human *VEGF*<sub>165</sub> not only improved neovascularization and hemodynamic recovery, but also resulted in better clinical outcome; i.e., limb amputation was reduced by 64% as compared with controls. Notably, the approach allowed a reduction of the dose of EPCs needed to achieve limb salvage by 30 times. Thus, combining EPC cell therapy with *VEGF* gene therapy may be one option by which the limitations imposed by paucity and dysfunctional activity of EPCs may be overcome. Several important questions, however, remain unsettled. It is not clear if transplanted EPCs enhance angiogenesis, vasculogenesis, or both. In addition, Iwaguro et al. failed to define the fate and possible side effects of systemic EPC delivery, including pathological angiogenesis in tissues distant from ischemic limb. A side-by-side comparison of *VEGF*-transduced EPCs vs direct *VEGF* gene transfer should help to understand the relative value of the new combinatory strategy.

Loss of telomerase activity has been suggested to activate the molecular mechanisms that lead to cellular senescence. Recently, Murosawa et al. demonstrated that EPC



**Fig. 7.** Representative graph of ex vivo gene therapy for therapeutic angiogenesis/vasculogenesis. (A) In vitro endothelial progenitor cells (EPCs) are cultured, expanded, and infected with adenovirus or other vectors encoding angiogenic genes (red). (B) Infected EPCs are further expanded and tested for their ability to release recombinant protein (violet). (C) Transfected EPC are injected into target tissue. (D) Some EPCs are destroyed after transplantation. (E) Surviving EPCs release angiogenic recombinant protein that in turn stimulates vessel growth (angiogenesis) from resident endothelial cells (ECs). (F) EPCs differentiate into mature ECs and form new vessels (vasculogenesis). (G) EPCs differentiate in myocytes or other mesenchymal cells under the influence of environmental stimuli. (H) EPCs holoclones can colonize targeted tissue, thus constituting a permanent reservoir of regenerative value in case of injury recurrence.

telomerase activity can be enhanced by transfection with human telomerase reverse transcriptase (*hTERT*) gene transfer (61). This resulted in improved migratory and mitogenic activity, better resistance to starvation-induced apoptosis, and augmented reparative neovascularization. The authors concluded that enhanced regenerative activity of EPCs by *hTERT* transfer may provide novel therapeutic strategy for postnatal neovascularization in severe ischemic disease patients.

Similar approaches could be used with chemokines and GFs. One possible target is the serine/threonine protein kinase Akt (also known as PKB), a key mediator of intracellular signal transduction processes. Overexpression of constitutively activated Akt mutants in many cell types promotes cellular transformation by two distinct mechanisms: (1) stimulation of proliferation under conditions in which cells should normally be growth-arrested; and (2) inhibition of apoptosis. Another interesting possibility is to use SDF-1 to augment EPC-mediated vasculogenesis. In vitro, expression of CXCR4, the receptor of

SDF-1, was reportedly detected in  $66 \pm 3\%$  of EPCs after 7-d culture (32). Furthermore, SDF-1 attenuates EPC apoptosis by 75%. In vivo, locally injected SDF-1 into athymic ischemic hind limb muscle of nude mice combined with human EPC transplantation increased local accumulation of fluorescence-labelled EPCs in ischemic muscle and increased capillarization and tissue perfusion recovery. It would be of paramount importance to see if SDF-1 transfection can trigger autocrine activation of EPCs and improve their viability.

Improvement of EPC engraftment could be obtained by silencing their apoptotic signaling. Techniques available to this aim include the use of antisense RNA, RNA interference and hammerhead, and hairpin RNAses.

## CONCLUSIONS

Therapeutic angiogenesis/vasculogenesis represents a new approach to treat patients with ischemic disease not curable with conventional treatment. Advances in our ability to genetically manipulate cells ex vivo has provided the technological platform to implement stem cell biology and circumvent the potential hazards of direct gene transfer. Ex vivo engineered endothelial progenitor cells have shown promising therapeutic profile for treatment of ischemia and for delivery of curative substances to target organs. Thus, genetic manipulation of stem cells represent a novel option for tissue regeneration.

## ACKNOWLEDGMENT

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## Cell Transplantation

*The New Frontier*

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### SUMMARY

Cell transplantation of noncontractile cells into ischemic or dilated cardiomyopathic hearts prevents progressive failure and improves cardiac function by incompletely understood mechanisms. Significant angiogenesis and extracellular matrix remodeling occur after cell transplantation, but these effects do not fully explain the improvement in systolic function of the heart. Recent provocative results suggest that cell transplantation may be able to induce neocardiomyogenesis, and may enhance regional systolic function by recruiting stem cells that differentiate into functioning cardiomyocytes. Enhancing these effects using a combined modality that includes transplantation of genetically modified cells may hold the key to future cardiac regeneration.

**Key Words:** Cardiomyopathy; cell transplantation; genetic modification; angiogenesis; stem cells; extracellular matrix.

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## INTRODUCTION

The discussion of cardiac cell transplantation within the context of a book dedicated to cardiovascular genomics is precarious but timely. Gene therapy evolved out of the central concept that providing one gene may reverse or prevent the progression of disease caused by the deficiency or altered function of a single gene. Intense investigation into the application of gene therapy has not only provided us with the opportunity to improve genetic techniques, genetic engineering, and gene delivery, but has taught us about the biology of single-gene disease processes, as outlined in the other chapters of this book. The process, however, has also highlighted the limitations of single-gene therapy. Even in diseases where the disease process is solely attributed to a single-gene defect, such as cystic fibrosis, gene-therapy strategies have yet to prove their clinical efficacy. Furthermore, the majority of the disease burden in the developed world is not caused by single-gene deficiencies but by degenerative disease processes that are largely multifactorial and polygenic. For instance, ischemic cardiomyopathy and heart failure are predicated on ongoing micro- and macrovascular atherosclerosis, micro- and macromyocardial infarctions and cell necrosis, ongoing myocardial ischemia and cell apoptosis, extracellular matrix remodeling with progressive left-ventricular dilatation, production of an inflammatory cytokine milieu, and distant effects, such as neurohormonal activation. Although gene therapy has been used to target a specific pathway in a complex degenerative process, such as angiogenesis in ischemic cardiomyopathy, the overexpression of a single gene late in the process will not be sufficient to halt it.

In a parallel to gene therapy, it was initially thought that if the principal insult that precipitates heart failure is myocyte death, then replacement of cardiomyocytes should reverse the heart failure phenotype. This original concept has now been significantly altered and revised. We and others have been unable to transplant cells that eventually beat in synchrony with the heart. In spite of this apparent major limitation, cell transplantation works (1). Various labs working with different animal models of heart failure, using a variety of cells have all, more or less, shown improvement in systolic function, prevention of ventricular dilatation, and prevention of heart failure. Albeit poorly understood, cell transplantation has begun to be enthusiastically investigated in human trials (2–6). The central question becomes: why should myocardial transplantation of noncontracting muscle cells or bone marrow cells prevent heart failure? We propose that unlike gene therapy, cell therapy invokes multiple endogenous processes that are capable of halting degenerative myocardial failure, such as angiogenesis, extracellular matrix remodeling, and perhaps cardiomyogenesis by stem cell recruitment. If living tissue heals, cell transplantation may work by cellularizing a largely acellular scar and converting it to living tissue, thus permitting and potentiating the mobilization of endogenous repair mechanisms. Here, the field of cell transplantation into postmitotic organs, such as the heart, directly encroaches on the fields of stem cells and stem cell plasticity. Our attention will, therefore, be focused mostly on these new frontiers of myocardial regeneration.

### CELL TRANSPLANTATION PREPARES THE HEART FOR REPAIR

#### *Angiogenesis*

When the field of myocardial cell transplantation was in its infancy, investigators did not evaluate the extent of neovascularization caused by cell transplantation because they did not expect it. Now it is well-known that a major effect of cell engraftment is the

induction of angiogenesis (Table 1), perhaps by the release of cytokines and growth factors, the participation of the cells in new vessel formation, and the recruitment of endothelial and smooth muscle cell precursors, progenitors, and/or stem cells. Compared with gene or protein angiogenic therapies, cell transplantation not only provides the signals for new vessels to grow into the ischemic region, but the donor cells provide the necessary cellular building blocks for new vessel formation in a region where cells are likely scarce. Angiogenesis in the absence of myogenesis may not influence overall heart function, but improving perfusion to the infarct region may itself modify the remodeling of both the injured region and the remaining myocardium. Increasing perfusion may salvage hibernating native cardiomyocytes and/or restore damaged cells. Increased perfusion may also aid in the restoration of injured matrix that in turn may facilitate donor cell incorporation, donor-to-host cell communications, and increased structural support and stability. This section highlights some of the advances that have occurred in investigating the effects of differentiated, progenitor, and stem cell transplantation on angiogenesis (capillary formation) and vasculogenesis (muscular vessel formation).

Considering the cell-type dependency of this process, the data suggests that cell transplantation-induced vasculogenesis is independent of the process of inflammation. We now know that vasculogenesis involves endothelial cell budding, endothelial progenitor cell (EPC) homing and differentiation, and bone marrow mesenchymal stem cell (MSC) mobilization and differentiation to smooth muscle cells to form the muscular media of the new vessels. The reviewed literature should be read, with the following serving as an underlying central question: why should cell transplantation cause vasculogenesis?

#### **TRANSPLANTATION OF DIFFERENTIATED CELLS**

Smooth muscle cell transplantation causes neovascularization in injured myocardium. Smooth muscle cells, under pathologic conditions, can de-differentiate, migrate, proliferate, and secrete extracellular matrix to heal vascular injuries. They also secrete factors, such as nitric oxide, basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) involved in the angiogenic process (7–9). Li et al. (10) successfully transplanted smooth muscle cells into the cryo-necrosed left ventricle of adult rats and observed a four- to fivefold increase in blood vessel formation, and significant improvement in contractile function. In a pig model of myocardial infarction followed by transplantation of uterine smooth muscle cells, we recently showed extensive formation of de novo muscular blood vessels (unpublished observation). Transplantation of vascular smooth muscle cells in the same model did not induce a similar degree of arteriolar and venular blood vessel formation. The uterine smooth muscle cells improved myocardial function more than the vascular smooth muscle cells, suggesting that part of the benefit of cell transplantation arose from its effects on vasculogenesis.

Likewise, albeit to a more limited degree, transplantation of skeletal myoblasts is also able to induce angiogenesis. Skeletal myoblasts are skeletal muscle precursor cells that have the capacity for self renewal and differentiation. Since the first experiment reported by Chiu et al. (11) of the successful engraftment of skeletal myoblasts, several studies have assessed the ability of these cells to improve postinfarction cardiac function (12–14). Initial studies did not demonstrate significant increase in angiogenesis caused by myoblast transplantation (15). However, in a study evaluating VEGF-transfected skeletal myoblasts, Suzuki et al. (16) showed that implantation of nontransfected skeletal myoblasts still resulted in significantly increased capillary number.

Table 1  
Cell Transplantation and Neovascularization

<i>Cells</i>	<i>Strategy</i>	<i>Results</i>	<i>Ref.</i>
Fetal rat stomach SMC Skeletal myoblasts + VEGF	IM, 4 wk after cryo injury, rats IM, 1 h after MI, rats	4 wk: increased capillary density 4 wk: increased capillary density in VEGF transfected > nontransfected > medium	10 102
EPCs	IM, 4 wk after MI, pigs	4 wk: increased capillary density. Improvement of myocardial function	31
EPCs	IC, 5 d after MI, humans	4 mo: improvement of coronary flow reserve	3
BMD-MC	IM, 4 wk after MI, pigs	4 wk: increased endothelial cell number and myocardial perfusion	32
BMD-MC	TCS, 2 wk after MI, pigs	2 wk: increased angiogenesis	33
BMD-MC	IM, after creation of a coronary stenosis, rats	2 mo: increased capillary density, blood flow, angiotensin 1 and VEGF expression	34
BMD-MC	IM, 60 min after MI, pigs	3 wk: increased capillary density, blood flow, and angiographic collateral vessels	37
BMD-MC	IC, 5–9 d after MI, humans	3 mo: improvement of myocardial perfusion	5

(continued)

BMD-MC	IM, ICM, humans	3 mo: improvement of myocardial perfusion	103
BMD-MC	IM, ICM, humans	2 mo: improvement of myocardial perfusion	104
HSC	IM, 5 h after MI, mice	9 d: donor-derived endothelial cells and SMCs in <i>de novo</i> capillaries and arterioles. Donor-derived cardiomyocytes	42
MSC	Intraaortic, 2 wk after MI, rats	4 wk: donor-derived endothelial cells, cardiomyocytes and fibroblasts	46
MSCs treated with 5-aza	IM, 3 wk after cryo injury, rats	8 wk: increased capillary density	105
MSCs treated with 5-aza	IM injection 4 wk after MI, pigs	4 wk: increased capillary density	106
MSCs treated with 5-aza	IM injection of noninjured heart, mice	1, 4, 8, and 12 wk: increased capillary density. Donor-derived endothelial cells	50

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SMC, smooth muscle cells; EPC, endothelial progenitor cells; BMD-MC, bone marrow-derived mononuclear cells; HSC, hematopoietic stem cells; MSC, mesenchymal stem cells; 5-aza, 5-azacytidine; IM, intramyocardial; IC, intracoronary; TCS, transcoronary sinus; MI, myocardial infarction; ICM, ischemic cardiomyopathy.

Recently, we investigated the enhancement of cell transplantation-mediated angiogenesis/vasculogenesis by transfecting the implanted cells with VEGF (17). We had hypothesized that over-expression of VEGF would enhance the beneficial effects of cell transplantation. Surprisingly, in our initial experiments, the greater degree of angiogenesis did not correlate with improvement in myocardial function (17). These observations are akin to our observations that endothelial cell transplantation caused significant angiogenesis but did not improve function (18). As such, increased angiogenesis does not necessarily imply greater functional benefit, whereas the process of vasculogenesis (as seen in our uterine smooth muscle cell experiments) appears to be directly correlated with functional improvement. Interestingly, our most recent results suggest that VEGF-enhanced cell transplantation causes significant upregulation of Flk-1 and Flt-1, VEGF receptors, in the infarct border zone (manuscript in preparation). That is, the VEGF transgene, which should only be highly expressed in the middle of the infarcted scar where the cells were transplanted, caused an upregulation of its receptors in the nontransplanted host cells located in the infarct border zone. This observation demonstrates for the first time that cell transplantation can affect host cells through both a paracrine, and perhaps a matrix-mediated, mechanism, and that cross-talk between the host and transplanted cells occurs.

#### TRANSPLANTATION OF PROGENITOR AND STEM CELLS

EPCs are bone marrow-derived progenitor cells that are implicated in the maintenance of endothelium through their ability to mature into endothelial cells and to support the process of angiogenesis (19). They have a common precursor with hematopoietic stem cells (HSCs) (20) and can be isolated from bone marrow or peripheral blood mononuclear cells (21–23). These cells express both the endothelial cell marker VEGF-receptor-2, and progenitor cell markers CD34 and/or CD133 (24,25). The number of circulating EPCs in the peripheral blood of humans is low under normal conditions, but can be significantly increased by bone marrow mobilization with granulocyte-colony stimulating factor (G-CSF) and VEGF (26,27).

Recently, Kaushal et al. (28) showed that *in vitro* expanded EPCs were able to recreate functional small vessels or cause angiogenesis. Human EPCs, when infused into athymic nude rats after myocardial infarction, induced significant angiogenesis, reduced host cardiomyocyte apoptosis, and prevented adverse remodeling (29,30). Kawamoto et al. (31) recently reported a significant increase in capillary density, angiographic collateral development, and left-ventricular ejection fraction 4 wk after transplantation of autologous EPCs in a swine model of myocardial infarction. Similarly, Kocher et al. (30) also showed that infusion of human EPCs in rats after myocardial infarction resulted in vasculogenesis. A recent clinical trial conducted by Assmus et al. (3) reported the feasibility and safety of intracoronary infusion of autologous EPCs after myocardial infarction in humans. Their preliminary evidence also suggests an increase in the perfusion of the implanted area.

The above studies clearly implicate EPCs in neovascularization. The possibility that differentiated cell transplantation also causes mobilization and homing of EPCs to the transplanted site requires investigation.

It appears that induction of angiogenesis is not limited to circulating EPCs because unselected bone marrow resident cells can also induce significant angiogenesis (32–34). Bone marrow contains many cell types that include MSCs and hematopoietic stem cells



(HSCs). Bone marrow-derived progenitors have the capacity to home to different tissue, proliferate, and acquire the phenotypes of the host organ (35,36). Kamihata et al. (37) reported that 16% of the bone marrow-derived mononuclear cells (BMD-MCs) that they implanted in a swine model of myocardial infarction expressed bFGF, VEGF, and angiopoietin 1, suggesting that the cells are precursors or progenitors of endothelial-like cells. Orlic et al. (35) reported that bone marrow cells mobilized by stem cell factor (SCF) and G-CSF also participated in angiogenesis. In the TOPCARE-AMI trial, Assmus et al. (3) observed a similar beneficial effect of unselected BMD-MCs and EPCs on normalization of coronary flow reserve. These results suggest either that the bone marrow cell preparations are “contaminated” with significant number of EPCs, or that other bone marrow-derived cells are also able to participate in the process of angiogenesis.

Evidence suggests that HSCs may participate in angiogenesis. HSCs have been shown to transdifferentiate to multiple lineages (38) (reviewed later). HSC participation in neovascularization is somewhat anticipated because they are mobilized by angiogenic factors such as VEGF and angiopoietin-1 (39,40), and have a common precursor with the EPCs, the hemangioblast (41). Indeed, Orlic et al. (42) observed a significant degree of neovascularization after transplantation of HSCs into the infarct border zone in a murine model. Jackson et al. (43) similarly reported that 3% of endothelial cells in ischemic myocardium expressed the marker of donor cells after bone marrow transplantation in lethally irradiated mice.

The other stem cell population that resides in the bone marrow, the MSC, also has been shown to significantly increase neovascularization after engraftment into injured myocardium (44–46). MSCs are a self-renewing clonal precursor of nonhematopoietic tissues that provide the microenvironment for hematopoiesis. They can be induced to differentiate into cells of mesenchymal lineage including fibroblasts, fat, cartilage, bone, skeletal, and cardiac muscle (47–49). Human MSCs do not express markers of EPCs and are not reported to transdifferentiate into endothelial cells (48,49). However, Gojo et al. (50) recently reported that immortalized murine MSCs treated *in vitro* with 5-azacytidine were able to transdifferentiate into endothelial cells, pericytes, and smooth muscle cells. Dr. C. Verfaillie’s laboratory (51) showed that the culture-adherent portion of bone marrow contains a subset of cells—multipotent adult progenitor cells—that capably participated in the generation of all adult mouse tissues when injected into the blastocyst. These cells express the VEGF receptors Flk1 and other phenotypic markers of endothelial lineage after VEGF stimulation. The contribution of MSCs, which are traditionally selected as the culture-dish adherent cells of the bone marrow, to neovascularization may be accounted for by the presence of the multipotent adult progenitor cells in the preparations.

Thus, several subsets of bone marrow cells, circulating progenitor cells, and differentiated cells are capable of inducing significant angiogenesis. These observations argue that a common mechanism must exist by which the various cell types induce angiogenesis and/or vasculogenesis. It appears that this mechanism is not cell-type-specific, but the extent of it depends on the cell type. Vasculogenesis requires the expression of various angiogenic cytokines and growth factors in addition to the recruitment of other cells, such as the EPCs and MSCs. It is unlikely that transplanted stomach smooth muscle cells, for instance, possess the machinery to independently induce significant angiogenesis. The cross-talk between the transplanted cells and the host cells is likely to be an important mediator. The challenge in the future will involve enhancing this cross-talk to induce the endogenous repair mechanisms that allow more efficient neovascularization.

### *Extracellular Matrix Remodeling*

Another beneficial effect of cell engraftment is the prevention of adverse matrix remodeling. The extracellular matrix provides the structural framework for coordinated cardiomyocyte contraction, and sequesters growth factors and cytokines. The myocardial matrix thus assembles a dynamic microenvironment in which molecular cues converge to maintain tissue architecture by regulating cell orientation, shape, growth, and survival (52–55). Maladaptive remodeling of the cardiac matrix impairs structural support for cardiomyocytes and leads to ventricular dilatation and both systolic and diastolic dysfunction (56,57). The engraftment of transplanted cells likely reorganizes the degraded matrix of the host myocardium and secretes new matrix elements, and may thereby regenerate normal myocardial tissue architecture. This process may also improve the structural support for native heart cells and, by tethering transplanted cells to actively contracting host tissue, may improve myocardial function. We are currently in the process of measuring the impact of cell transplantation on the extracellular matrix, both in the infarct zone as well as in the remote myocardium.

### *Myogenesis*

Considering that the transplanted cells significantly induce angiogenesis and may induce beneficial matrix remodeling, one wonders what other repair mechanisms are mobilized by the process of transplantation. Recent evidence suggests that injured or stressed myocardium is able to recruit cardiac-derived c-Kit<sup>+</sup> stem cells or bone marrow-derived c-Kit<sup>+</sup> hematopoietic stem cells (reviewed later). Therefore, mechanisms exist in the body to allow mobilization and homing of appropriate stem cells to the injured heart to give rise to an unknown degree of neocardiomyogenesis and neovascularization. It is possible that cell transplantation enhances the mobilization of such mechanisms that are obviously inadequate to prevent progressive heart failure in patients after a myocardial infarction. The aforementioned mechanisms may only be in place to replace the low level cell loss that occurs with physiologic apoptosis of cardiomyocytes, and are overwhelmed by massive cardiomyocyte loss. Cell duress may be responsible for mobilizing stem cells, and the massive amount of noncardiomyocyte cell duress that occurs with cell transplantation may significantly potentiate these endogenous repair mechanisms. The beneficial effects of matrix remodeling and vasculogenesis may act as adjunct mechanisms that allow, enhance, or aid these processes. We propose that cell transplantation amplifies not only the signals that promote stem cell mobilization and proliferation, but also their effects on extracellular matrix and angiogenesis. That is, the transplanted cells may pave the way for the successful engraftment of recruited cardiac and bone marrow stem cells.

## **BONE MARROW STEM CELLS AND STEM CELL PLASTICITY**

Traditionally, hematopoietic stem cells reside in the c-Kit<sup>+</sup>, Sca-1<sup>+</sup>, Thy<sup>lo</sup>, and Lin<sup>neg/low</sup> fraction of the bone marrow (58,59). Until recently, these cells were thought to only give rise to hematopoietic cells. Mice that bear a partial mutation in c-Kit-receptor tyrosine kinase activity are partially deficient in hematopoietic stem cells and readily accept bone marrow transplantation without prior lethal irradiation. Complete mutation of this transmembrane protein results in an embryonic lethal phenotype. The c-Kit receptor is a membrane-bound signaling tyrosine kinase expressed on HSCs, neural-crest-derived stem cells, and primordial germ cells, but not on mature hematopoietic cells (60). The c-Kit ligand,

(SCF), upon c-Kit binding, dimerizes the receptor with subsequent autophosphorylation and activation of intracellular signaling, leading to stem cell survival, proliferation, and mobilization (61,62).

Work from Dr. M. Goodell's laboratory at the Baylor College of Medicine has identified an alternate method of stem cell purification that has had an impact on identifying other tissue-resident stem cells. In a series of experiments, Goodell and colleagues were able to show that the Hoechst dye-excluding fraction of the bone marrow, the side population or SP cells, were able to stably reconstitute the bone marrow of irradiated recipients in long-term reconstitution experiments (63). Further work from the same lab suggested that the bone marrow SP cells have the potential to differentiate into cardiomyocytes in ischemic myocardium (60). These observations, coupled with a myriad of investigations reporting that adult stem cells from the bone marrow can differentiate into non-lymphohematopoietic tissues, such as myocytes (64), hepatocytes (65), microglia and macroglia (66), and neuronal tissue (67,68), have given rise to the burgeoning field of "stem cell plasticity."

SP cells have also been isolated from muscle tissue. In fact, when these muscle SP cells from wild-type donors were transplanted into *mdx* mice, a mouse model of muscular dystrophy, new muscle cells were noted to express the dystrophin gene (69). These muscle cells must have been derived from the transplanted muscle SP cells. Furthermore, the muscle SP cells reconstituted the bone marrow of lethally irradiated hosts (70). Subsequent work demonstrated that muscle-resident SP cells were likely hematopoietic in origin (71). But why would hematopoietic stem cells reside in the muscle? Are they contaminating cells with no function, or do they exist in other tissues for tissue repair? And more interesting, why would muscle-resident hematopoietic stem cells be able to correct the genetic deficiency in muscle tissue? Last, if stem cells exist in skeletal muscle tissue, do stem cells exist within the fabric of cardiac muscle tissue?

Perhaps the most intriguing observation comes from investigation of neural stem cells. When grown in culture, these cells form spheroids and are capable of generating oligodendrocytes, neurons, and astrocytes. When transplanted into irradiated hosts, they too were capable of reconstituting the bone marrow (72). Work of other investigators suggests that the bone marrow cells are also capable of giving rise to neurons (68). Whether this process occurs by transdifferentiation, dedifferentiation followed by redifferentiation to another fate, or fusion of bone marrow cells with existing neurons is unclear, and is the subject of intense investigation.

The series of observations demonstrating that single cells from one tissue can give rise to cells of another tissue across germline boundaries prompted Dr. H. Blau (73) from Stanford University to publish an editorial in the journal *Cell* in which she argued that perhaps stemness is a cell function, and that stem cells are cells that perform that function. Central to her argument is the concept that most cells have the capability, under the appropriate condition, to be stem-like; that is, they have the capability of dedifferentiating, proliferating, and redifferentiating. These are tantalizing suggestions that beg the question: why would cells retain such activity, or is there a physiological role for stem cell plasticity? The theme that seems to be emerging is that stem cells are critical mediators of tissue response to injury.

### BONE MARROW STEM CELLS AND MYOCARDIAL INJURY

Whereas the heart was previously viewed as a postmitotic organ, recent evidence suggests that the myocardium has some replicative potential, and it may have the poten-

tial for self-repair or self-regeneration (74). Examination of cell division in both normal and pathological human myocardium revealed myocyte proliferation rates of 14 to 140 per million myocytes (75). In the infarct border zone, estimates of cardiomyocyte proliferation approached 4–5% of the total cardiomyocyte number (76).

Mobilization of the bone marrow-derived stem cells prior to the induction of myocardial infarction in a murine model led to significant engraftment of bone marrow-derived cells into the injured myocardium (35). Thus, myocardial injury is able to elicit the homing of bone marrow stem cells to the heart. Hematopoietic stem cells that are c-Kit<sup>+</sup> have also been shown to transdifferentiate into cardiomyocyte when directly transplanted into injured murine myocardial tissue (77). The developing myocytes were small and resembled fetal and neonatal myocytes. They were positive for several myocyte-specific proteins, including cardiac myosin and connexin-43 and the transcription factors GATA-4, MEF2, and Csx/Nkx2.5 (77). Jackson and coworkers demonstrated, using lethal irradiation and bone marrow reconstitution with labeled cells, that 0.02% of new cardiomyocyte and 3% of new endothelial cells after myocardial infarction are from the bone marrow (43).

Evidence from humans also implicates the bone marrow as a potential source of cardiomyogenesis. Muller et al. (78), Laflamme et al. (79), and Quaini et al. (80) observed that male heart transplant recipients who had received a female heart had cardiomyocytes bearing the Y-chromosome, thus suggesting that cardiomyogenic precursors had been recruited from an extracardiac location. Conversely, cardiomyocytes bearing the Y-chromosome were discovered in female patients who had received bone marrow transplantation from male donors (81), implicating the bone marrow as the extra-cardiac source of cardiomyogenic precursors.

Other models of tissue injury and repair have also highlighted the central role of bone marrow-derived cells. Sata et al. (82) demonstrated that the majority of smooth muscle cells involved in postangioplasty restenosis, graft vasculopathy, and hyperlipidemia-induced atherosclerosis were from the bone marrow. We also found, in a femoral angioplasty model in irradiated mice reconstituted with HSC from transgenic yellow fluorescent protein mice, that most smooth muscle cells within the intimal hyperplastic lesion had yellow fluorescence. This observation suggests that bone marrow-derived cells had migrated to the site of injury and had transdifferentiated into or fused with resident smooth muscle cells. Additionally, formation of intimal hyperplasia after vascular injury was absent in mouse models that bore a mutation in the signaling domain of c-Kit or that lacked the membrane isotype of its ligand, stem cell factor (manuscript in preparation).

Evidence outside the realm of cardiovascular medicine has also implicated bone marrow cells as important mediators of tissue regeneration. Models of bone marrow transplantation in animals with fumarylacetoacetate hydrolase deficiency have demonstrated significant engraftment of bone marrow-derived cells into liver parenchyma (83,84). These cells produce albumin and phenotypically appear to be hepatocytes. Interestingly, evidence from the two separate labs also demonstrated that the new hepatocytes were the result of fusion between the bone marrow-derived cells and hepatocytes (83,84). The new heterokaryons (products of cell fusion) expressed fumarylacetoacetate hydrolase. This latter observation suggests, as another editorial by Dr. H. Blau argues (85), that fusion may actually be a physiologic event by which damaged cells receive fresh genetic material that may restore function. That is, fusion may be a mechanism by

which epigenetic changes associated with a diseased phenotype are “reset”, or new genetic functionality is added.

The mechanism of stem cell mobilization and homing to the injured organ is becoming increasingly elucidated, and some of these data are summarized below. The signals appear to consist of molecules involved in stem cell mobilization and release, migration, homing, and engraftment, the principle of which appears to be mediated through the c-Kit receptor.

An important signal appears to come from binding of the Kit receptor with its ligand, SCF. Lyman et al. (86) investigated the role of and the mechanism by which SCF mobilizes bone marrow-derived stem cells. Bone marrow suppression induced the expression of matrix metalloproteinase-9 (MMP-9) that caused the cleavage and release of SCF from the membrane of bone marrow cells, which in turn enhanced hematopoietic reconstitution by binding the kit receptor on the surface of the hematopoietic stem cells and promoted release and active proliferation of these cells. Furthermore, chemokine-induced mobilization of bone marrow repopulating cells by stromal-derived factor, VEGF, and G-CSF was impaired in MMP-9<sup>-/-</sup> animals.

SCF also appears to play a major role in peripheral mobilization of bone marrow cells. SCF administered to mice for 7 d resulted in the depletion of c-Kit<sup>+</sup> Sca<sup>+</sup> Thy<sup>lo</sup> Lin<sup>-</sup> bone marrow stem cells in the bone marrow, but the multilineage, long-term reconstituting activity in the spleen and peripheral blood increased proportionally (87). These observations suggest that systemic SCF administration caused redistribution of existing stem cells to peripheral sites, making them available for tissue repair. SCF may also be an important homing signal for the bone marrow-derived hematopoietic progenitor cells (88). Indeed, administration of antibodies which block c-Kit resulted in reduced homing efficiency of progenitor cells (89). Other investigators have shown that SCF also induces expression of very late antigen family of integrins, which mediate the adhesion of hematopoietic progenitor cell lines to fibronectin and vascular cell adhesion molecule-1 (90–93). These proteins may play a critical role in the adherence and transmigration through the endothelial barrier of the HSC into injured tissue.

Recent findings suggest a role for SCF in myocardial injury as well, although this role needs to be fully elucidated. In a canine model of ischemia/reperfusion, Frangogiannis et al. (94) demonstrated upregulation of SCF mRNA 72 h postischemia. One possible mechanism is the aiding of mobilization and homing of c-Kit<sup>+</sup> stem cells. The investigators identified a subset of tissue macrophages as the source for the SCF, thereby linking inflammation to stem cell mobilization. Considering the central role of antigen-presenting cell that tissue macrophages play in the immune system, one may hypothesize that these cells, recruited as part of the inflammatory response to any site of injury, can then initiate/propagate either an immune response or a repair response in a dichotomous fashion.

## CARDIAC-DERIVED STEM CELLS

The hypothesis that the heart is also home to cardiac-specific stem cells that are capable of cardiomyogenesis is supported by recent evidence. Many terminally differentiated tissues have been shown to harbor cells with stem cell-like activity (95). We have previously isolated myofibroblasts from the heart, which can be expanded *ex vivo* and will prevent functional deterioration when implanted into an infarcted region. Other investigators found a different cell population in the heart, SP cells, that may be cardiac-

specific stem cells (96). These cells were first identified, as detailed previously, in the bone marrow and were shown to have a high percentage of known HSC surface markers: c-Kit<sup>+</sup>, Sca-1<sup>+</sup>, CD43<sup>+</sup>, and CD45<sup>+</sup>. Hierlihy and colleagues (96) have been successful in isolating SP cells from single-cell preparations of murine heart. They showed that when the growth of the postnatal heart was attenuated in the dominant negative MEF2Cdn (cardiac-specific transcription factor) murine hearts, the SP cell population was depleted as the number of cardiomyocytes increased significantly. The authors were unable, however, to produce a direct link between SP cells and increased cardiomyogenesis.

Dr. M. D. Schneider's group from the Baylor College of Medicine recently provided the missing link connecting the myocardial SP cell population to myocardial regeneration (97). A critical attribute of cells capable of self-renewal and extended proliferative capacity, as one would expect from stem cell or progenitor cells, is the capacity to prevent telomere shortening that leads to cell senescence. The Schneider group was able to identify a subset of the myocardial SP cell population as the cells that expressed 100% of the telomerase activity resident in the myocardium. Interestingly, the cell population was the Sca-1 positive subpopulation of myocardial SP cells. Characterization of these cells using a combination of gene-expression profiling, reverse-transcription polymerase chain reaction (RT-PCR), and protein expression analysis confirmed that these cells expressed a number, but not a complete set, of cardiac-specific transcription factors. The cells, however, did not express cardiac-specific structural proteins. These cells, when cultured in the presence of the demethylating agent 5-azacytidine, acquired the capability to differentiate to cardiomyocytes. When injected intravenously after an infarction, the cells homed to the infarct border zone and gave rise to new cardiomyocytes (97).

The work of Dr. P. Anversa's group, from the New York Medical College, has recently proved the existence of cardiac-resident stem cells that appear to be less differentiated than the cells isolated by Schneider's group (98). Examination of myocardial tissue from old rats revealed the presence of small c-Kit<sup>+</sup> cells that otherwise lacked markers of blood-derived cells. These round cells with little cytoplasm were isolated and grown *in vitro* at a single cell level. The resulting colonies formed spheroids that remained c-Kit<sup>+</sup> and undifferentiated, but also gave rise to satellite cells that became adherent to the culture dish. Immunohistochemistry demonstrated the production of smooth muscle actin, sarcomeric actin, and vWF—indicative of smooth muscle, cardiomyocyte, and endothelial differentiation, respectively—in a different subset of the satellite cells. These cells slowly lost their c-Kit expression and, as verified by the expression of cardiac-specific transcription factors and structural genes, readily formed cardiomyocytes. The transplantation of these cells into the infarct border zone, after myocardial infarction, ameliorated myocardial dysfunction induced by cell death, and over time allowed the synchronized beating of the transplanted area, as evidenced by echocardiography (98).

The same group demonstrated that similar c-Kit<sup>+</sup> cells undergo rigorous cell proliferation in the left-ventricular biopsy samples of patients who had come to aortic valve replacement because of severe aortic stenosis (99). Interestingly, in that study, the duration of symptomatic heart failure corresponded inversely to the number of dividing c-Kit<sup>+</sup> cells and directly to the degree of telomere shortening. These findings suggested, as would be expected from the limited capability of myocardial regeneration after injury, that the purported cardiac stem cells did not have unlimited proliferative capacity. These observations open the door, if the above findings are reproduced in magnitude by other groups, for strategies that use *ex vivo* expansion of these cells prior to implantation to prevent ongoing degeneration of myocardium.

However, we do not know the physiological role that these cardiac-derived stem cells play in response to myocardial infarction and progressive heart failure. Although the previously noted human studies, in addition to the data from rats, suggest an important role, the magnitude, time-line, and extent of the repair process caused by these cells remain unknown. The observation that these cells are not present uniformly in the myocardium (they are mainly concentrated in the atrial tissue, as well as in the left-ventricular apex) would also suggest some sort of mechanism for migration of these cells. These cells may either migrate through the interstitial space, or be mobilized into the blood stream with subsequent homing to the injured myocardium. If these cells are similar to hematopoietic stem cells, as suggested by phenotypic characterization and cell-surface protein expression, they may retain a similar capacity to be released from their immediate niche into the circulation. As suggested by chimeric bone marrow studies, these cells should also have the capacity to breach endothelial barriers and transmigrate back into myocardial tissue to undertake myocardial repair.

### GENE-ENHANCED CELL TRANSPLANTATION TO REGENERATE THE HEART

Thus, the fields of cell transplantation and stem cell biology meet to provide promise for attempts at cardiac regeneration. The possibility that stem or progenitor cells are recruited to the area of cell transplantation where injury signals are once again generated by the transplanted cells may be the major mechanism by which cell transplantation of various cell types may impact cardiac function to a similar degree. Delineating the precise molecular mechanisms by which these cells are mobilized and recruited, and the subsequent signaling pathways that promote differentiation or transdifferentiation of the recruited cells to the cardiomyocyte lineage, are and will be areas of intense investigation. It is within this context that genetic modification of transplanted cells may hold significant potential (Table 2).

Gene-enhanced cell transplantation is still in its infancy, but already impressive results of its potential have arisen in the literature. Dr. V. J. Dzau's group from Harvard Medical School transfected rat MSCs with the *AKT1* gene, which is a serine-threonine kinase and is involved in survival signaling (100). Transplantation of the transfected cell into the infarct zone within an hour of coronary ligation resulted in near normalization of both systolic and diastolic function in a dose-dependent manner at follow-up. Interestingly, the transplanted MSCs appeared to have transdifferentiated into cardiomyocyte-like cells. Furthermore, these cells appeared to express connexin 43 and N-cadherin at junctions with host cardiomyocytes. It is unclear from their data whether the cells had truly transdifferentiated, or had fused with native cardiomyocytes.

Recent evidence from the Cleveland Clinic has highlighted another utility of gene-enhanced cell transplantation. Askari and colleagues conducted a series of experiments that for the first time provided evidence for the recruitment of c-Kit<sup>+</sup> cells to the site of cell transplantation (101). Chronic BrdU labeling after myocardial infarction resulted in the labeling of bone marrow cells and not cardiomyocytes. Eight weeks after myocardial infarction, and after BrdU administration was halted, transplantation of skeletal myoblasts resulted in an increase in the BrdU signal in the heart, suggesting that previously-labeled cells were recruited to the site of transplantation. Transfection of cardiac fibroblasts with stromal-derived factor-1 (SDF-1), a known stimulator of stem-cell homing which is expressed for up to 7 d after infarction (101), and transplantation of these

Table 2  
Protein- or Gene-Enhanced Cell Transplantation

<i>Cells</i>	<i>Strategy</i>	<i>Results</i>	<i>Ref.</i>
Fetal rat CM + bFGF microspheres	MI, rat	4 wk: improved function in combination therapy	107
Neonatal rat CM + HGF liposomes	MI, rat	4, 8 wk: increased angiogenesis and improved function in combination therapy	108
VEGF transfected rat adult CM	Cryo injury, rat	5 wk: increased angiogenesis but no improvement in function	17
VEGF transfected rat skeletal myoblasts	MI, rat	2 wk: reduced infarct size and improved function	102
CTLA4-Ig transfected rat fetal CM	Normal myocardium, mice	8 wk: reduced xenograft rejection	109
Akt transfected rat neonatal CM	Cryo injury, rat	4 d: increased survival of cells	110
Akt transfected rat MSC	MI, rat	2 ws: reduced infarct size and improved function	100
IGF transfected rat SMC	Cryo injury, rat	1 wk: increased survival of cells and increased angiogenesis	9
SDF-1 transfected Rat cariac fibroblasts and G-CSF admin	MI, rat	4 wk: increased homing of BM c-Kit <sup>+</sup> cells and improved function	101

CM, cardiomyocytes; bFGF, basic fibroblast growth factor; HGF, hepatocyte growth factor; VEGF, vascular endothelial growth factor; CTLA4-Ig, cytotoxic T lymphocyte late antigen-4 immunoglobulin; IGF, insulin-like growth factor; SMC, smooth muscle cells; SDF-1, stromal derived factor-1;



cells into the infarct region, resulted in a significant increase in the BrdU signal in the heart and improvement in cardiac function. Interestingly, a significant number of these cells were either CD34<sup>-</sup> or c-Kit<sup>+</sup>, suggesting that they were endothelial progenitor cells or c-Kit<sup>+</sup> stem cells, although proof of this hypothesis was not provided.

## CONCLUSION

Although cell transplantation was initially developed to directly replace lost cardiomyocytes after injury, the transplanted cardiomyocytes did not beat synchronously with the rest of the heart. We have discovered that the transplanted cells have a multitude of other effects that may account for the functional benefit seen with this therapeutic strategy. Angiogenesis and vasculogenesis occur to a significant degree in the recipient heart. We are also beginning to understand the extent of the influence of the transplanted cells on extracellular matrix remodeling. The role of cardiac-resident or bone marrow-derived stem cells in myocardial response to injury is becoming increasingly elucidated. The mobilization of the endogenous repair mechanisms that may involve stem cell recruitment may be a major mechanism by which cell transplantation arrests progressive heart failure. Once we understand the cross-talk signals that drive these mechanisms, we may be able to intervene and not only arrest progression of disease, but perhaps reverse it. A combination of cell- and gene-therapy may hold the future key to cardiac regeneration.

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## Embryonic Stem Cells and the Cardiovascular System

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### SUMMARY

Human embryonic stem (ES) cells are pluripotent cells isolated from the inner cell mass of blastocyst-stage embryos. These cells are capable of self-renewal and can differentiate into many cell types. In vivo, human ES cells injected into immune-deficient mice yield teratomas with ectodermal, mesodermal, and endodermal cell derivatives. In vitro, spontaneous aggregation of human ES cells results in the formation of embryoid bodies (EBs) comprised of differentiated cells from the three embryonic germ layers. The addition of growth factors can induce the differentiation of human ES cells into specific populations of cells, among them the cells of the cardiovascular system, cardiomyocytes, and endothelial cells. Functional cardiomyocytes caused the EBs to exhibit rhythmic contractions. The cells of the cardiovascular system were distinguished by molecular markers and by their structural and functional characteristics. Human ES cells may serve as a source of cells for cellular transplantation in different pathologies, among them cardiovascular diseases, and as a model to study the embryonic development of human beings.

**Key Words:** Embryonic stem cells; differentiation; cardiomyocytes; endothelial; cardiovascular system.

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## INTRODUCTION

Embryonic stem (ES) cells are line of cells derived from the inner cell mass (ICM) of blastocysts. ES cells were first isolated from mouse embryos (1,2). The pluripotency of these cells is demonstrated by the use of three models: (1) embryoid body (EB)—a structure that is formed by the spontaneous aggregation of ES cells in suspension. The cells that comprise this EB propagate and differentiate, resulting in a cavitated structure that causes the EB to become cystic. It was shown that this cystic EB consists of cells from the three embryonic germ layers (3). (2) Upon injection of the ES cells into immune-deficient mice, the cells form teratomas. These teratomas are differentiated tumors comprising many cell types (3). (3) Chimeric mouse embryos may be achieved by injecting ES cells into blastocysts. The injected ES cells contribute to all embryonic tissues including the germ cells (4). Various methods of genetically manipulating mouse ES cells were performed for the purpose of mutating an endogenous gene or introducing a transgene. This mouse ES cell system enabled the studying of interesting genes *in vitro*, but more importantly the creation of chimeras is an extremely useful tool for the analyzing of genes during the embryo development *in vivo*.

In this chapter, we will discuss the isolation of human ES cells, their spontaneous and directed differentiation, and the genetic manipulations induced in these cells. We will devote special attention to the differentiation of human ES cells into cells of the cardiovascular system.

## DERIVATION AND CHARACTERIZATION OF HUMAN EMBRYONIC STEM CELLS

Human ES cells were derived from spare cleavage-stage embryos of *in vitro* fertilized eggs, grown to the blastocyst stage (5,6). Cells of the inner cells mass from these blastocysts were plated onto mitotically inactivated murine embryonic fibroblast feeder cells, and formed compact colonies of human ES cells. It was shown that these cells have a normal karyotype even when they are grown for an extended period of time in culture (5,6). Human ES cells were found to have high activity levels of telomerase (5), a protein responsible for maintaining the chromosome length and that is tightly correlated with immortality in human cell lines (7). Undifferentiated human ES cells are characterized by specific cell surface markers and enzymatic activities. For example, the stage-specific embryonic antigens 3 and 4 and the markers TRA-1-60 and TRA-1-81 are expressed in human ES cells and are downregulated during their differentiation (5). Pluripotency is defined as the ability of the cells to differentiate into cells comprising the three embryonic germ layers, namely, ectoderm, mesoderm, and endoderm. The pluripotency of human ES cells was proven both *in vivo* and *in vitro*. *In vivo*, injection of the human ES cells into immune-deficient mice generated teratomas harboring derivatives of all three embryonic germ layers (5). *In vitro* aggregation of human ES cells brought about the formation of EBs (8). The cells within the EBs acquired molecular markers specific for the three embryonic germ layers. The differentiating cells also acquired characteristic morphologies and further developed new functions. The functionality of cells, such as cardiomyocytes, is evident from the appearance of pulsing muscle cells (8). Human ES cells were clonally derived either by dilution or by genetic selection in order to show the pluripotency of the single cells (9,10). The different clonal cells also exhibited the creation of EBs that contained cells of the three embryonic germ layers.

## DIFFERENTIATION OF HUMAN EMBRYONIC STEM CELLS

Growing the human ES cells in suspension caused the cells to aggregate and form EBs. This process was similar to that found in mouse ES cells (8). Initially, the clusters of ES cells are composed of densely packed cells, creating simple EBs. After several days, the center of the bodies become cavitated and they begin to accumulate fluid and develop into cystic EBs. The cells within the EBs were shown to express  $\alpha$ -fetoprotein,  $\zeta$ -globin, or neurofilament 68 kd—molecular markers specific for the three embryonic germ layers (8). In addition, rhythmic pulsation was observed, and cardiac muscle cells expressing  $\alpha$ -cardiac actin were also demonstrated in these human EBs. Cells with various morphologies were revealed as a result of the dissociation of EBs and the plating of the differentiated cells as a monolayer (11). The differentiation through EBs is spontaneous and seems stochastic. The effects of eight growth factors on human ES cells were assessed in order to direct the differentiation of the cells (11). Analysis of cell morphology and the expression pattern of a large number of cell-specific markers confirmed the ability to induce specific differentiation by various growth factors. Thus, activin-A and transforming growth factor (TGF)- $\beta$  mainly induce mesodermal cells; retinoic acid (RA), epidermal growth factor (EGF), bone morphogenetic protein 4, and basic fibroblast growth factor activate ectodermal and mesodermal markers; and nerve growth factor and hepatocyte growth factor allow differentiation into the three embryonic germ layers, including endoderm. Initial protocols were also established in order to induce the directed differentiation of human ES cells. For example, epidermal skin cells were enriched in the presence of EGF, myocardial cells were achieved by addition of TGF- $\beta$ , and cartilage differentiation was evident when the cells were treated with bone morphogenetic protein 4 (11). Further analysis showed that more molecular markers appeared upon differentiation of human ES cells, verifying the existence of many cell types such as hematopoietic cells (12) and cells with characteristics of insulin-secreting  $\beta$ -cells (13). The differentiation of neuronal progenitor cells may be achieved even without the creation of EBs, because early stages of neuroectodermal differentiation were found in very dense cultures of human ES cells (6). In serum-free media, the cells that were isolated from the culture created spherical structures that expressed early neuronal markers, and when plated and grown as a monolayer, these cells expressed more mature neuronal markers (6). Neuronal differentiation may be induced by growth factors; thus, Schuldiner et al. (11) showed the ability of RA or nerve growth factor to induce neuronal differentiation under specific conditions. Moreover, RA supported the production of mature neurons that express dopamine or serotonin receptors, and the formation of complex plexuses of neuronal cells.

## GENETIC MANIPULATION OF HUMAN EMBRYONIC STEM CELLS

Genetic manipulations of ES cells enable the introduction of foreign genes, such as marker or master genes, and the mutagenesis of endogenous genes. Marker genes are very useful for studying the expression profile of a gene during differentiation and for tracing and selecting a particular subset of the cells (14,15). The overexpression of master genes in mice, such as certain transcription factors, can lead to differentiation into specific cell lineages (16,17). The ability to mutate specific endogenous genes, either by homologous recombination or by gene trapping, changed the face of mammalian genetics (18). Chimerism using ES cells allows germ line transmission of a transgene, thus enabling in vivo

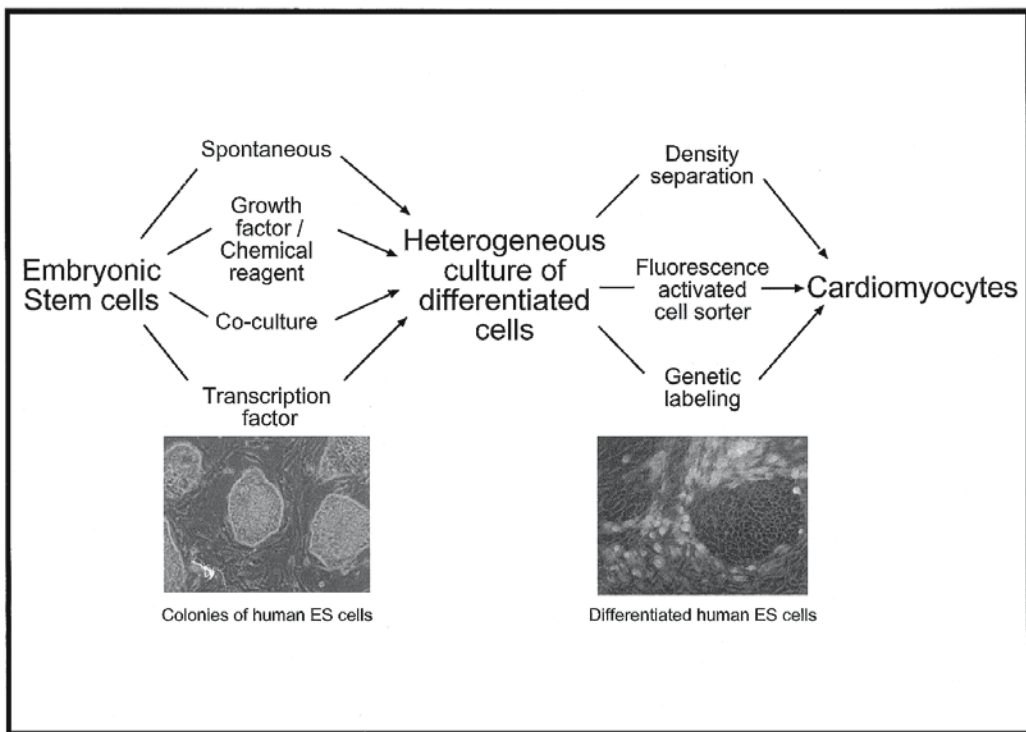


testing of the effect of the transgene or the mutation. The mutant knockout mice shed new light on gene function and enable the creation of many mouse models of human genetic diseases (19).

Eiges et al. (10) established a DNA transfection protocol for human ES cells that is different from the one used for murine ES cells. Human ES cells were also transduced by a lentiviral vector, resulting in the infection of almost 100% of the cells (20). The transfection of human ES cells with a reporter gene was the first attempt at utilizing genetic manipulation of cells in order to address a biological issue. Human ES cells were transfected with green fluorescent protein (GFP) while being controlled by Rex 1, an ES cell-enriched gene. The transfected cells showed high levels of GFP expression, which were limited to the undifferentiated cells, and a fluorescence-activated cell sorter assisted in separating the differentiated cells from the fluorescent cells (10). This way, it was possible to sort out undifferentiated, potentially tumorigenic cells in order to prevent the risk of transformation of the cells during transplantation. In addition, the use of genetic manipulation in human ES cells should allow us to follow and select a desired subset of differentiated cells from a heterogeneous culture, thus purifying only a specific type of cells prior to transplantation. Protocols for homologous recombination in human ES cells, which were developed only lately, enable us to study gene function in humans more thoroughly (21). Figure 1 shows various ways of differentiating human ES cells and methods for selecting specific subsets of cells.

## CARDIOMYOCYTES AND ENDOTHELIAL CELLS DERIVED FROM HUMAN ES CELLS

One of the most impressive evidences of differentiation of human ES cells is the creation of pulsing embryoid bodies that contain myocardial cells (8). The creation of spontaneously beating cardiomyocytes in mice was concomitant with rhythmic action potentials very similar to those described for embryonic cardiomyocytes and sinus-node cells (22). During formation of EBs, cardiomyocytes derived from mouse ES cells exhibit typical signal transduction pathways through adrenoceptors and cholinceptors. Alterations in contractile sensitivity to  $Ca^{2+}$  during ES differentiation in culture provided functional evidence that cardiomyocytes derived from ES cells recapitulate embryonic cardiogenesis (23). Several distinct cell populations could be distinguished during cardiomyocyte differentiation through characteristic sets of ionic channels and typical action potentials, presumably representing cardiac tissues with properties of sinus node, atrium, or ventricle (24). Overall, the developmental program of mouse ES cells towards cardiomyocytes resembles that observed during cardiogenesis *in vivo* (25). Pure myocyte culture from differentiated mouse ES cells was established using the antibiotic selection of ES cells carrying sequences encoding for antibiotic resistance under the control of  $\alpha$ -cardiac myosin heavy chain promoter. The selected cells were able to form stable intracardiac grafts in the hearts of adult dystrophic mice (14). The previously mentioned selection method was recently combined with a large-scale process, using stirred suspension cultures and deriving higher amounts of purified cardiomyocytes from mouse ES cells (26). Nkx2.5 is a transcription factor that plays a fundamental role in the transcriptional regulation of cardiac-specific genes. Lately, a Nkx2.5/GFP<sup>+</sup> mouse ES cell line was established by knocking in an endothelial GFP reporter gene into the Nkx2.5 locus. These cells were proven to be precursor cells of sinoatrial node, atrial or ventricular cell types (27).



**Fig. 1.** Differentiation and selection of embryonic stem (ES) cells. Schematic representation of various ways to differentiate ES cells and methods for purifying specific subset of cells. Also shown are colonies of human ES cells on a feeder layer of mouse embryonic fibroblasts and heterogeneous population of nondifferentiated and differentiated green fluorescent protein (GFP)-positive human ES cells.

Cardiomyocyte loss in the adult mammalian heart may be irreversible, and frequently leads to diminished cardiac function. The ability to increase the number of functional cardiomyocytes in a diseased heart would have obvious therapeutic potential. Recent studies have suggested that cellular transplantation can be used to increase the number of myocytes in cardiac pathologies. Even though several different types of myocyte preparations were successfully engrafted in animals, it is difficult to obtain a sufficient number of donor cells in humans (28). ES cells may serve as an alternative renewable source of donor myocytes. Rhythmic pulsation of myocytes was observed in cystic human EBs, and *in situ* hybridization with a marker of embryonic myocardial cells revealed that the central cavity of the pulsing EB was in fact surrounded by cardiomyocytes (8). In addition, cells from the spontaneously contracting areas were positive to many cardiac-specific genes and transcription factors that are involved in cardiogenesis (29,30). Morphologically, the cells presented varying degrees of myofibrillar organization, which were mainly consistent with early stage cardiomyocytes. Electrophysiological tests presented a relatively long action potential duration, characteristic of cardiomyocytes that are significantly different from noncardiac muscle, and the cells also presented normal chronotropic effects to drugs (30). Furthermore, it may be possible to achieve induction of differentiation into cardiomyocytes by treating the ES cells with

Table 1  
The Properties of Cardiomyocytes Derived From Human Embryonic Stem Cells

<i>Methodology</i>		<i>Evidence for active cardiomyocytes</i>
Molecular markers	RNA	$\alpha$ -cardiac actin (8,11); atrial myosin light chain, ventricular myosin light chain, $\alpha$ -myosin heavy chain, atrial natriuretic peptide, cardiac troponin T and I, GATA-4, Nkx2.5 (30); $\alpha$ -myosin heavy chain, atrial natriuretic factor, Nkx2.5 (32)
	Protein	$\alpha$ , $\beta$ -myosin heavy chain, cardiac troponin I, desmin, atrial natriuretic peptide, $\alpha$ -actinin (30); $\alpha$ -actinin (29) $\alpha$ , $\beta$ -myosin heavy chain, cardiac troponin I and T, sMHC, desmin, tropomyosin, $\alpha$ -actinin, GATA4, MEF2, N-cadherin, creatine kinase-MB, myoglobin, $\alpha$ 1 and $\beta$ 1 adrenoreceptors (32)
Morphology and physiology	Cell morphology	Mononuclear and round or rod-shape (30)
	Electron microscopy	Immature phenotype of disorganized myofibrillar stacks in early stage EBs and more organized sarcomeric structure in older EBs, also Z bands and intercalated disks (30), poor sarcomeric banding pattern (29)
	Rhythmic contractions	30 pulses/min (8); 8.1% of 22-d-old EBs contained contracting areas, usually in the outgrowth of the EB with $94 \pm 33$ pulses/min (30); 60 pulses/min (29); up to 70% of the EBs contained contracting areas, rate of $40 \pm 10$ pulses/min (32)
	Electrophysiology	Relatively long action potential duration characteristic of cardiomyocytes (30)
	Pharmacological studies	Positive and negative chronotropic responses were observed (30), ion blocker decreased the contraction rate, adrenoreceptors agonists enhanced contraction rate (32)
Induction of differentiation	Growth factors	Induction of differentiation by TGF- $\beta$ (11) 5-aza-dC, a demethylation reagent, enhanced cardiomyocytes differentiation (32)
	Coculture	Induction of differentiation by mouse visceral endoderm-like cell line End-2 (29,31)
Enrichment of cardiomyocytes	Percoll gradients	Fourfold enrichment from 17 to 70% cardiomyocytes using percoll gradient separation (32)

MEF, murine embryonic fibroblast; TGF, transforming growth factor; EB, embryoid body.

TGF- $\beta$  (11), or co-culturing the cells with END-2 cells (visceral endoderm-like cells similar to those normally adjacent to the region of heart development) (29,31). Treatment of the cells with 5-aza-2'-deoxycytidine, a demethylation reagent, could also enhance cardiomyocyte differentiation, which was shown to be enriched by Percoll density centrifugation to give a population containing 70% cardiomyocytes (32). The functional

heterogeneity of cardiomyocytes derived from human ES cells was demonstrated by typical action potential of nodal-like, embryonic atrial-like, and ventricular-like cardiomyocytes in different outgrowths of beating EBs (33). Table 1 presents the phenotypic and functional properties of cardiomyocytes derived from human ES cells.

Increasing the number of cardiomyocytes requires induction of new vascular structures. Tissue engineering approaches have already reported the production of vascular graft material from smooth muscle and endothelial cells (34). Endothelial progenitors were also shown to have a therapeutic potential for myocardial ischemia (35). Mouse ES cells may differentiate into endothelial (36,37) and smooth muscle cells (38,39), and serve as a potential source of cells to form vascular structures. Yamashita et al. (40) established a system for induction and purification of vascular progenitors (flk1+ cells) from mouse ES cells. Flk1, one of the receptors for vascular endothelial growth factor, is a marker for lateral plate mesoderm (41), and the earliest differentiation marker for endothelial and blood cells (42,43). Indeed, the flk1+ cells gave rise to two major vascular cell types (endothelial cells and smooth muscle cells) *in vitro* (36,44) and *in vivo* (40).

Endothelial cells were also derived from human ES cells. These cells were isolated from 13- to 15-d-old EBs by using platelet endothelial cell-adhesion molecule-1 (PECAM1) antibodies, and further characterized *in vitro* and *in vivo* (45). PECAM1 is a marker for mouse embryonic endothelial cells (46) and is expressed in human EBs in correlation with vascular endothelial-cadherin (45,47), suggesting that it could serve as a marker for human embryonic endothelial cells as well. The endothelial cells were found in groups within these EBs, organized in specific channel-like structures, and thus may show spontaneous differentiation to endothelial cells and blood vessel-like structures. *In vivo*, when PECAM1+ cells were transplanted into immune-deficient mice, the cells appeared to form microvessels. Some of the human vessels had mouse blood cells in their lumen, suggesting that they had become functional (45).

The accumulating data on the differentiation of human ES cells into the different populations of cells within the heart suggests that these cells may harbor a huge therapeutic potential. Thus, in the future, differentiated human ES cells may be used as an ultimate source of cells for transplantation cardiovascular medicine.

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