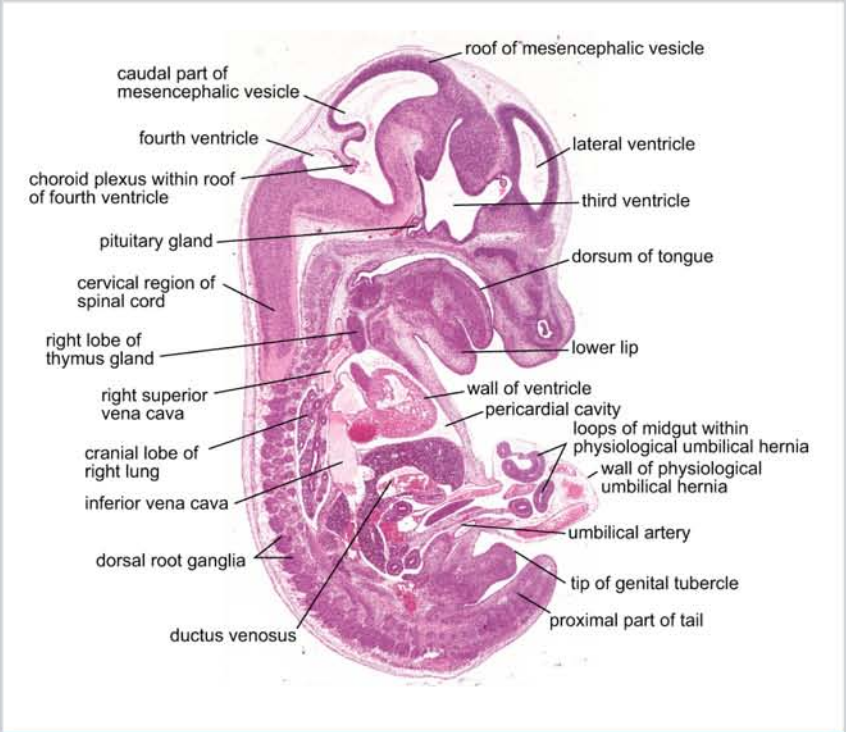
Histologic Basis of Mouse Endocrine System Development

A Comparative Analysis



Matthew Kaufman
Alexander Yu. Nikitin
John P. Sundberg

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A Comparative Analysis

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Preface

Dr. Kaufman created the classic textbooks on mouse embryology that remain heavily used to this day and will continue to be used for years to come. Unfortunately, the technology at the time was black-and-white photomicrographs and line diagrams. While these are superb, they are small and do not provide the detail needed by today's mouse pathologists. To address this problem, we took advantage of the new digital "virtual slide" technology to scan the best images in Dr. Kaufman's collection to generate color images at various magnifications. While the focus of this book is on comparative embryology of the endocrine organs, the embryonic images at various developmental stages contain many other organs. The representative images are provided in the text for orientation and reference. The accompanying DVD designed by Dr. Nikitin provides the virtual slides that can be viewed on your computer screen in color and at various magnifications. Installing the ImageScope™ software (Aperio Technologies, Inc.) to access the files turns your computer into a "virtual microscope" where the image from the glass microscope slide can be moved across the screen and areas of interest enlarged for more detailed evaluation. This tool allows the reader to look at specific organs or structures at various magnifications at different stages of embryogenesis, making this a unique tool to help identify structures in normal mouse embryos while comparing with embryos under investigation. The text integrates historical perspectives of embryogenesis of the endocrine system between many species, but primarily between humans and mice. The digital images focus entirely on the mouse as this is intended to be an adjunct to assist with analysis of genetically engineered mice with embryonic abnormalities. The main text and histologic sections were prepared by Dr. Kaufman and, where considered necessary, the text has been amended and updated by Drs. Nikitin and Sundberg. The idea of this book was proposed by Dr. Sundberg during the first annual Practical Workshop Series on the Pathology of Mouse Models for Human Disease in the Jackson Laboratory in 2002.

Acknowledgments

We are grateful to Drs. Jinhyang Choi, Elena N. Schmidt, Zongxiang Zhou, and Mr. David C. Corney in Dr. Nikitin's laboratory for their dedicated work on preparing digital slides of Dr. Kaufman's histologic sections with the ScanScope® system (Aperio Technologies, Inc., Vista, CA). We thank Jesse Hammer at the Jackson Laboratory Audiovisual Services for final labeling of the images; the label text was provided by Dr. Kaufman. We also thank Maxine Friend and Norma Buckley for their assistance with reference formatting.

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We owe a special debt of gratitude to our wives, Claire Kaufman, Andrea Flesken-Nikitin, and Beth A. Sundberg, for their continuous support, encouragement, and patience.

The Authors

Matthew H. Kaufman, M.D., Ch.B., Ph.D., D.Sc. was appointed in 2007 as emeritus professor at the University of Edinburgh in Scotland. He was professor of anatomy from 1985 to 2007 at the University of Edinburgh. In 2008 he was elected F.R.S. Edinburgh, having in 1996 been elected F.R.C.S. Edinburgh and F.R.C.P. Edinburgh in 2000. He initially graduated in medicine from the University of Edinburgh in 1967 and practiced for a number of years, most recently in obstetrics and gynecology. In 1973 he gained his Ph.D. in physiology from the University of Cambridge and then undertook a postdoctoral post from 1973 to 1975 at the Weizmann Institute in Israel. He then returned to Cambridge to a junior lectureship and then to lectureship in anatomy. His Ph.D. and postdoctoral studies were predominantly on parthenogenetic development in the mouse. His subsequent research work has principally been in the field of developmental biology. In 1979–1980 he was involved with Dr. Martin Evans in the establishment for the first time of pluripotent stem cells in tissue culture. His parthenogenetic studies were complemented by the publication of his text *Early Mammalian Development: Parthenogenetic Studies* in 1983. From 1980 to 1985 he was elected a fellow of King's College in Cambridge. He returned to Edinburgh and was chair of anatomy in 1985, and his research from that time on has mainly been in the field of clinical embryology. Since he moved to Edinburgh he has collaborated with developmental biologists and computer scientists based in the Medical Research Council (MRC) Human Genetics Unit to prepare 3D reconstructions of many of the stages of mouse development illustrated in his text *The Atlas of Mouse Development*, first printed in 1992. This was complemented by his *The Anatomical Basis of Mouse Development* (co-authored with Dr. J. B. L. Bard), published in 1999. Most of his research in Edinburgh has related to the analysis of mouse models for human birth defects, principally investigating the influence of chromosomal constitution, particularly the role of ploidy, ethanol, and anesthetics. Since 2001 he has published numerous papers and six books (with an additional book in press) on various aspects of medical history: three on historical aspects of military surgery, one on medical teaching in Edinburgh during the 18th and 19th centuries, and one on phrenology and biographies of Dr. John Barclay (1758–1826) and Robert Liston (1794–1847, in press). He currently lectures worldwide on mouse embryology.

Alexander Yu. Nikitin, M.D., Ph.D. is associate professor of pathology at Cornell University College of Veterinary Medicine in Ithaca, New York. He received his M.D. with a distinction from the the I.P. Pavlov State Medical University of St. Petersburg (formerly the I.P. Pavlov First Leningrad Medical Institute), Russia, in 1983 and was a staff scientist and diagnostic pathologist at the Professor N. N. Petrov Research Institute of Oncology in St. Petersburg, Russia, until 1988. He received his Ph.D. in pathology from the same institution in 1988 and undertook postdoctoral training in cell biology at the Institute of Cell Biology (Cancer Research) at

the University of Essen Medical School in Essen, Germany, and in molecular biology at the Institute of Biotechnology at the University of Texas Health Science Center at San Antonio (UTHSCSA) in Texas. During his postdoctoral studies he was among the first investigators to generate transgenic rats and to demonstrate that mice heterozygous for the retinoblastoma (*Rb*) gene develop a syndrome of multiple endocrine neoplasia. After holding instructor and research assistant professor positions at UTHSCSA, he joined Cornell University faculty as assistant professor of pathology in 2000. Dr. Nikitin was promoted to associate professor with indefinite tenure in 2007 and was named leader of the Cornell Stem Cell Program in 2008. Dr. Nikitin was vice chair of the Pathology and Laboratory Medicine Standing Committee of the Mouse Models of Human Cancers Consortium from 1999 to 2004 and a recipient of the National Center for Research Resources (NCRR) of the National Institutes of Health (NIH) Midcareer Award in Mouse Pathobiology in 2002–2007. He has been a cofounder and a co-organizer of the NCRR-supported Annual Practical Workshop on the Pathology of Mouse Models for Human Disease held at multiple locations in the United States since 2002. Dr. Nikitin is associate editor for the journal *Cancer Research* and has been a member of the Academy of Genomic Pathology since 2007. During his entire career, Dr. Nikitin has been interested in the application of animal modeling, cell and molecular biology to solving questions of cancer pathogenesis. Dr. Nikitin established a number of new mouse models of human cancer including the first autochthonous mouse model of serous ovarian adenocarcinoma, models of metastatic prostate cancer, mammary cancer, neuroendocrine neoplasia, and soft-tissue sarcomas. Most recently he demonstrated that prostate carcinomas associated with deficiency for tumor suppressors p53 and Rb arise from the prostate stem cell compartment. His laboratory also was among the first to identify microRNAs of miR-34 family as targets of p53. Dr. Nikitin has published more than 70 research articles and 7 book chapters. Current research interests of Dr. Nikitin's laboratory focus on the role of stem cell compartment in carcinogenesis, identification of early determinants of metastatic progression, and development of more accurate mouse models for human cancer.

John P. Sundberg, D.V.M., Ph.D., Dipl. A.C.V.P., received a B.S. degree in agriculture and animal science from the University of Vermont in Burlington in 1973 and his doctor of veterinary medicine degree from Purdue University School of Veterinary Medicine in West Lafayette, Indiana, in 1977. He entered a private large (food) animal practice in Meadville, Pennsylvania, after graduation. Dr. Sundberg completed a residency in anatomic pathology and a Ph.D. in virology and comparative pathology in the Department of Pathobiology at the University of Connecticut in Storrs in 1981. From there he became assistant professor of pathology in the College of Veterinary Medicine at the University of Illinois in Champaign. He passed the veterinary anatomic pathology specialty board exam in 1982. At the University of Illinois he continued his thesis research on the role of papillomaviruses in cancer where he developed the dog oral papillomavirus model. His lab cloned and sequenced the canine oral papillomavirus genome. Building on these tools, he and colleagues at Georgetown University School of Medicine created a recombinant vaccine that provided the first proof of concept for a papillomavirus vaccine that ultimately resulted in development of the human cervical cancer vaccine. In 1986 he became associate professor at the Jackson Laboratory in Bar Harbor, Maine, becoming a full professor in 1998. Because the Jackson Laboratory is infectious disease-free and maintains strict barriers to exclude infectious diseases, Dr. Sundberg changed his primary research focus to phenotyping mutant laboratory mice, developing a mouse pathology program, and a long-term program to create mouse models for genetic-based skin diseases. Concurrently, he continued the vaccine studies as collaborations at other institutions. Dr. Sundberg is an established leader in

the field of mouse pathology, especially mouse models of diseases of the skin and adnexa. He has published more than 300 articles in major medical journals, 125 book chapters, and 7 books on mouse pathology, biomethodology, and models for biomedical research. He was one of the first recipients of the NIH NCRR K26 mid-career awards for mouse pathobiology (2000–2006). He organizes meetings on the pathology of mouse models of human diseases that are held throughout the United States annually. He is a cofounder and a co-organizer of the NCRR-supported Annual Practical Workshop on the Pathology of Mouse Models for Human Disease held at multiple locations in the United States since 2002 (along with Dr. Nikitin) as well as cofounder of the larger international Pathology of Genetically Engineered Mice Meetings held since 1999. These meetings set the standards for mouse pathology meetings held today by many groups. The interaction among the faculty members of the workshop resulted in the collaborative production of this book.

1 Introduction

The principal aim of this book is to describe the embryological development of the various organs that constitute the endocrine system in the human and rat for comparison with the mouse. The mouse has been selected, as this has proved to be an extremely useful species for providing an increasing number of models of the wide range of disease states known to affect this organ system in the human. The glands that comprise the primary endocrine system are diversely located throughout the body and consist of the following: the pituitary, thyroid, parathyroid, adrenal, pancreas, pineal gland, as well as the ovary and testis. While morphologically they appear to have relatively little in common, they all share a common physiological role, in that they are involved in the expression of a variety of genes that are associated with the control of the secretion of a series of polypeptide hormones. Their common feature is that they control the development but, more particularly, the function of many of the other organs of the body. The hormones that are produced by these organs are themselves controlled by complex feedback mechanisms and by this means are associated with the maintenance of homeostasis. In addition to the complex structure of the endocrine glands, the "classical" endocrine system, many organs contain trophic cells with endocrine function scattered throughout their parenchyma. As the focus of this book is embryonic and anatomic embryogenesis and development of the endocrine glands, these highly specialized cells in specific organs will not be covered here. These will be covered in future books covering specific parenchymous organs.

While it is recognized that the full gestation period of the mouse is substantially shorter than that of the human, the early development of the mouse has probably been studied in considerably greater detail than comparable events in the latter species. This is principally because of the wider accessibility of both pre- and early post-implantation stages of mouse embryonic development and the relative ease to which it has been possible to undertake experimental studies using both intact embryos and more recently genetically manipulated cell lines that are derived from them. While it was formerly technically possible to observe the development of the pre- and early post-implantation stages of mouse development only in tissue culture, this is now no longer the case. The availability of pluripotent cell lines established from the inner cell mass region of blastocysts and, more recently, from primordial germ cells, has allowed the development in tissue culture of a very wide range of cell types. These pluripotent cell lines have provided an essential step toward development of technologies allowing specific modification of mouse genes and led to the 2007 Nobel Prize in Physiology or Medicine. These approaches led to rapid progress in creation of genetically engineered mouse (GEM) models that now allow for the analysis of normal and an increasingly wide range of diseases. Despite the enormous advances in this area, it is still essential to understand the early events that are associated with the normal development of the various endocrine glands in the mouse. During the early stages of their development, all of the endocrine organs appear to form without any obvious common features. It is only later, once a reasonable state of differentiation has been achieved, that it is possible to recognize that they, in fact, appear to share certain common features. All appear to be associated with a well-developed vascular system, and this is later seen to be of critical importance in facilitating their homeostatic role in the adult. With regard to the pineal gland, in particular, this possesses complex neural connections with the retina and, some authorities believe, additionally to a wide range of other structures.

The complete gestation period of the mouse lasts only about 19.5 to 21 days and is accordingly far less than that in the human. It is also relevant to note that the end of the so-called embryonic period in this species occurs at a stage comparable to about 13.5 days of gestation in the mouse. Despite the fact that the process of organogenesis in the mouse is essentially completed in most

organ systems by about 15.5 days of gestation, the overall degree of development achieved is, for obvious reasons, far less than that observed in the human embryo at the time of birth. This is because of the considerable period of "consolidation" seen in the human fetus during the second half of gestation, for which no comparable period is present in the mouse. It is also for this reason that it is necessary in the present volume to complement the information obtained from the analysis of mouse embryonic development with additional information obtained from the analysis of both pre- and early post-pubertal mouse, rat, and human material.

As indicated above, while most of the information described here will relate to the early stages of the development of the endocrine organs in the mouse, where necessary, attention will be drawn to the obvious similarities and differences between the morphological features observed in the two major species of interest: mouse and human. As far more information is presently available on the histopathological features of human—principally adult—disease states, it is likely that it will take many years before a comparable nucleus of information is available for the mouse. Indeed, for the very wide range of subtle disorders, particularly of the nervous system, it has to be appreciated that it may never be possible to recognize comparable clinical states in the mouse, although in recent years a variety of novel tools have been developed to analyze mouse neurophysiology and behavior.[1–3] It is for this reason that at some stage in the future there may well be limitations to the value of this species in a considerable range of biomedical research studies, although for each obstacle, solutions continue to be found. Despite this reservation, it is likely that the mouse will continue to provide an invaluable source of information for a considerable proportion of disease states studied for the foreseeable future. In relation to experimental studies on certain glands, where no mouse embryonic or even adult information is available, important studies have been undertaken on other rodent species, and reference will be made to these studies in the present text.

As indicated previously, the mouse remains the most commonly used and important animal model for assisting in the understanding of the disease states observed in the human. It is equally important as a model system for understanding the disease states observed in a range of other species. It is beyond the scope of this book to systematically discuss diseases of the endocrine system. These are described in a variety of textbooks,[4–13] and specific genetic based models can be found online (http://www.informatics.jax.org; http://www.ncbi.nlm.nih.gov/omim/). For these reasons, it will be necessary to employ specially trained pathologists to interpret the mouse material involved in these studies. Some of these pathologists will clearly be trained in human pathology, while the majority are likely to be in the field of veterinary pathology. During both the medical and veterinary courses, an increasing amount of time needs to be spent during the training period in learning about the *normal* anatomical features of the relevant species studied and the complexities of their physiology.

Accordingly, because of curricular changes, and because less individuals are appropriately qualified to teach these disciplines than was previously the case, combined with the introduction of other disciplines into the curriculum, relatively less time is available now than formerly to consider the embryological development of the various species studied. Similarly, less time is available than formerly in the clinical component of these courses to study the histopathology of the various organ systems, but particularly those that constitute the endocrine system. Over the last few years, it has become increasingly necessary for subspecialists to devote much of their time to the interpretation of serial sections of the relatively early stages of normal mouse development. Only when these sections are thoroughly understood is it possible to interpret comparable sections of the early stages of heterozygous and homozygous mutant mouse embryos.

It also follows that as the general curriculum expands, so the amount of time available to consider specific aspects of the course diminishes. This has inevitably led to the need for individuals to specialize in areas that were formerly covered by generalists. In the field of endocrinology, for example, there has recently been an expansion in the number of erudite multiauthored textbooks and multiauthored chapters that deal specifically with this topic.[14,15] While formerly a few individuals felt able to cover the entire field, this has not been the case for many years. One reason for this clearly relates to the new and improved diagnostic techniques that have been developed within this

discipline, and particularly the availability of specialized molecular methodologies that necessitate the large number of authors not infrequently observed in these volumes.[14]

The main aim of the present volume is therefore to provide a series of representative figures that display the histological features of hematoxylin and eosin stained sections of the various endocrine organs at sequential stages of their development in the mouse. The range of stages shown in each section of the text will consequently display the principal features that are observed in a particular organ from the earliest stages of its development to those seen at the time of birth in the mouse. While there is variation between strains, the B6CBA F1 hybrid was used as a reference strain. Many of the physiological and eventually anatomical spectra among inbred, recombinant inbred, and some hybrid strains are available online (www.phenome.jax.org). All of the histologic sections shown will be appropriately labeled to draw attention to their principal anatomic features. Where necessary, and if available, these sections will also be complemented by similar sections stained to display other features not readily seen in hematoxylin and eosin stained material. Each section will also be associated with a detailed figure legend and be complemented by a descriptive text that will aim to emphasize the progressive and sequential changes observed in the postnatal development of these organs. Accordingly, any evidence of *delayed* development or differentiation that is observed in the reader's own sections prepared from embryos isolated from timed matings will also be immediately apparent. It will also be necessary to complement the prenatal mouse material with comparable material from pre- and early post-pubertal mice in order to cover the stages of development observed in human early postnatal material.

In the text, attention will also be drawn to complementary information obtained from other sources. This may include information derived, for example, from the analysis of appropriate gene expression patterns where this is available from the relevant literature. The emphasis in this volume will be largely toward the preparation of a descriptive "atlas" or "workshop guide" of the developmental anatomy of the various organs that comprise the endocrine system during the period between the first evidence of the differentiation of a particular organ and birth in the mouse complemented by stages comparable to the situation observed at birth in the human. Consequently, there is likely to be relatively little information on any relationship that may exist between structure and function, as few if any experiments have been undertaken to investigate the functional capacity of these organs during these prenatal stages of development. It is also for this reason that the majority of the available information presented here is likely to be of a purely descriptive nature. We provide with this book an attached DVD containing digital "virtual slides" that were used to generate all histological images in this text. These slides were generated using a ScanScope® Scanner (Aperio Technologies, Inc., Vista, CA). Using the ImageScope™ software (Aperio Technologies, Inc.) the entire embryonic section can be viewed, scanned, and magnified to enable the user complete evaluation of the sample as if the actual slide were being viewed. While numerous websites (http://www.civm.duhs.duke.edu/devatlas/index.html; http://genex.hgu.mrc.ac.uk/Atlas/intro.html; http://genex.hgu.mrc.ac.uk/; http://mouseatlas.caltech.edu/) provide embryonic data generated using modern imaging technologies, such as nuclear magnetic resonance imaging, these still cannot provide the detail of hematoxylin and eosin (H&E) stained slides of whole embryos that are serially sectioned.

BACKGROUND

In any consideration of the endocrine system, it is necessary not only to draw attention to the overall control of this system but also to consider how the various organs that together constitute this system interact to control homeostasis. Possibly of greatest importance involves the control of anterior pituitary hormone secretion by the hypothalamus, although attention was drawn to the critical neural relationships that exist between the pineal gland and other closely related structures. General observations on the clinical relationship between the pituitary gland and the hypothalamus have long been known. This is due, for example, to the influence of certain anterior pituitary tumors

on the excess accumulation of subcutaneous fat and associated hypogonadism long recognized as Fröhlich's syndrome (http://cancerweb.ncl.ac.uk/cgi-bin/omd?Frohlich's+syndrome).[16]

It is now also appreciated that the hypothalamus controls the anterior pituitary gland via the pituitary portal venous system. By this means, the median eminence of the hypothalamus is now known to be closely associated with the anterior part of the pituitary gland. As a result of numerous largely experimental studies that were principally undertaken on early postnatal stages of development, it now appears clear that the hypothalamus controls the anterior pituitary indirectly via chemotransmitters that are mediated through the hypothalamo-pituitary portal venous system. Of critical importance would appear to be an understanding of the role of certain releasing factors produced by the hypothalamus and how they act via the pituitary gland. By this means, the control of a wide range of bodily functions is achieved. For example, these releasing factors are now known to play a critical role in the control of reproduction, growth, and development as well as in the maintenance of fluid balance and the control of the body's response to stress. These functions are all achieved through the control of pituitary hormone production by the hypothalamus. For recent observations on the interrelationship between the hypothalamus and pituitary and how neural control of glandular secretion is achieved, see Cone et al.[17]

The intimate relationship that is established between the hypothalamus and pituitary gland occurs at a very early stage of mammalian development, and how this arises in the mouse is considered in detail in the first section of the text.

GENERAL APPROACH TAKEN IN THIS VOLUME

While the text in each chapter provides an overview of the information available in the appropriate literature, the illustrations of the histologically sectioned material, as indicated above, were obtained almost exclusively from the analysis of serially sectioned hematoxylin and eosin stained embryos. While the majority of these embryos were sectioned in the sagittal plane, some were sectioned in the transverse plane. While the majority of the sagittally sectioned embryos were embedded in paraffin and cut at a nominal thickness of about 4 μm, others were sectioned at about 5 μm (the developmentally less advanced embryos) or between 4 μm and 7–8 μm (in the case of the developmentally more advanced embryos and early postnatal stages of development). This applied equally to all of the transversely sectioned embryos. Information relating to the nominal thickness and plane of section of all of the illustrations is provided in the associated figure legends. It should also be noted that the convention used in this text is similar to that employed in Dr. Kaufman's previous volumes on mouse embryology.[18] Accordingly, the stages of mouse development up to the time of birth are referred to as *embryos*, despite the fact that from about embryonic day E13.5 (13.5) to term the stages studied are comparable to *fetal* stages of early human development. Similarly, the morning of finding a vaginal plug in the mated females, none of whom were induced with exogenous hormones, is referred to as E0.5 of gestation.

In the accompanying DVD, all digital slides are located in folders named by chapter number and organ name (e.g., 2_Adrenal). The slide names consist of the earliest embryonic day (E) and figure number in the book. For example, virtual slide of mouse embryo used for imaging of the adrenal gland at E13.5–l4 and represented by Figure 2.1 is labeled "E13.5_Fig2.1.svs." The names of these slides are also indicated in the figure legends of the book in parentheses immediately after the figure number, for example, Figure 2.1 (E13.5_Fig2.1.svs). Please see the ReadMe.txt file on the DVD for additional instructions.

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2 The Adrenal (Suprarenal) Gland

INTRODUCTION

Two adrenal glands are present in the adult, each located just rostral and medial to the upper poles of the kidneys. Each adrenal gland possesses a fibrous capsule subjacent to which is a cortex of coelomic epithelial (mesodermal) origin, and a medulla principally of neural crest-derived sympathetic nerve (ectodermal) origin. Two early cell types are recognized in the medulla: the sympathoblasts, which give rise to the mature sympathetic ganglion cells, and the phaeochromoblasts, which develop into the chromaffin cells. The gland also has a rich blood supply. In the adult human, the suprarenal arteries, which are usually of a large size compared to these glands, are derived from the abdominal aorta and pass directly to the suprarenal glands. These arteries enter the gland after dividing into several small branches. Other branches to the adrenals, usually termed the middle suprarenal arteries, are of various diameters and arise laterally from each side of the aorta. They anastomose with the suprarenal branches of the phrenic and renal arteries. The inferior suprarenal arteries ascend to the suprarenal glands from the renal arteries. The suprarenal vein emerges from the hilus of the gland. The one on the right side drains into the inferior vena cava, while that on the left side drains into the left renal vein.[1,2] The arrangement observed in rodents has been described by Lever,[3] and had previously been discussed by Gersh and Grollman[4] and somewhat later by Harrison.[5] The arrangement observed in rodents and in a number of other mammalian species differs from that in the human, and is discussed in detail later in this chapter.

DEVELOPMENT OF THE ADRENAL GLAND IN THE HUMAN EMBRYO AND FETUS

Formation of the Adrenal Cortex

In the human embryo, the first evidence of cortical tissue is seen at about 28 to 30 days *post coitum* (dpc), in embryos of about 6 mm crown-rump (CR) length, at about Carnegie Stage (CS) 14.[6] At this stage of development, the first evidence of cortical differentiation of the future adrenal gland is located between the root of the dorsal mesentery of the stomach (the dorsal mesogastrium) and the medial part of the developing gonad that develops in the medial aspect of the mesonephric ridge. These columnar coelomic epithelial cells proliferate and form cords of tissue that invade the subjacent mesenchyme of the dorsal abdominal wall. By this means, a mass of discrete but substantial size consisting of large acidophilic cells is formed on either side of the midline dorsal aorta. Shortly afterwards this first collection of cells becomes surrounded by a second layer of smaller cells, and by this means forms an outer subcapsular layer. These smaller cells are later destined to form the definitive cortex of the gland. The larger cells form the so-called *fetal* or *primitive cortex*, and these regress shortly after birth.

When the embryo has a CR length of about 13 to 17 mm, at about 44 dpc, Crowder[6,7] noted that the cortical region of the gland became substantially reorganized, with the formation within it of

numbers of sinusoids. Substantial numbers of subcapsular cells, termed C1, C2, and C3, of coelomic epithelial origin, separated these. By about 48 dpc, it was noted that the C2 cells were now principally located on the surface of the gland, to form a distinctive capsule.[8] At about this time, Jirásek[9] noted that the cortex was also now invaded by a considerable number of cells of medullary origin, to form small "islets" of medullary tissue in this region. By CS 21, in embryos of about 22 to 24 mm CR length, at about 52 dpc, Crowder noted that the C2 cells that formed a capsule over the surface of the gland assumed a fibrous appearance.

In a study undertaken on 20 pairs of adrenal glands isolated from human fetuses between 9 and 38 weeks of gestation, the volume of different regions of the cortex and their average cell volume was determined.[10] The rate of increase in the weight of these glands was exponential during the period studied. This was particularly due to the enlargement of the fetal zone, with less marked changes observed in the other zones. While the zona glomerulosa (ZG) and outer part of the zona fasciculata (ZF) were recognized after the 20th week, the zona reticularis was not recognized in this material. Before 20 weeks of gestation, the volume of the ZF cells was considerably greater than the cells in the ZG but smaller than that of the cells in the fetal zone. After 20 weeks of gestation, the increase in volume in the fetal zone was much greater than in the other regions of the cortex. While the average volume of the cells in the ZG and ZF remained constant throughout the fetal period, those in the fetal cortex increased from the 9th to the 20th weeks of gestation, and then remained constant.

The growth of the fetal zone of the adrenal cortex is believed to be regulated by adrenocorticotrophic hormone (ACTH) during prenatal development, and it has been suggested that ACTH also stimulates the expression of locally produced growth factors such as insulin-like growth factor 2 (IGF2) and fibroblast growth factor beta (FGFB).[11] During development, the outer definitive zone of the cortex accumulates progenitor cells that later move inward to populate the rest of the cortex of the gland. It has been suggested that placenta-derived corticotrophin-releasing hormone, which is produced at high levels close to full-term, may be involved in inducing fetal adrenal steroidogenesis and the initiation of parturition. Shortly after birth, the fetal zone rapidly regresses, possibly due to the action of activin and transforming growth factor beta (TGFB).

By about CS 23, Crowder noted that the C2 cells that previously formed the fibrous capsule of the gland now invaded the gland. Its arterial blood supply arises from the adjacent mesonephric arteries, and these invade the gland in a radial manner. These then united with the venous sinusoids that had earlier been established in this region of the gland. The subcapsular cortical cells (possibly of C1 and C3 origin) now intermingle to form the *zona glomerulosa* (ZG) of the gland, and these cells and those from the capsule now migrate centrally as cords between the capillaries and sinusoids. The cells that constitute the cords degenerate and ultimately disappear during the first few weeks after birth. The loss of these cells, that constitutes the fetal cortex, is the cause of the marked reduction in the size of the gland that occurs at this time. According to Johannisson,[12] this constitutes about 80% of the fetal adrenal cortex at term. It is suggested that the fetal cortex probably synthesizes a considerable part of the estrogenic precursors that are eliminated in the maternal urine during pregnancy. It is also suggested that this region of the fetal adrenal may be one of the main users of placental progesterone required for the synthesis of adrenocortical hormones during this period. In this study,[12] the adrenal cortices isolated from a total of 42 human embryos and fetuses at various stages throughout pregnancy were examined by electron microscopy in order to determine the sequential changes in cortical morphology that occurred during this period. Twelve additional conceptuses were used to study the ultrastructural response of the adrenal cortices to the influence of various hormones, such as ACTH and HCG, and to gonadotrophin deprivation. The examination of the adrenal cortices of a number of anencephalic full-term fetuses revealed a lack of any steroidogenic activity, and it was speculated that this was probably due to a lack of ACTH activity, similar to that observed following hypophysectomy in experimental animals.[12]

Formation of the Adrenal Medulla

The medullary component of the adrenal gland is also first evident at about the 10-mm CR length stage, and this is formed principally from neural crest-derived sympathetic nerve fibers that grow out (the rami communicantes) from the paravertebral sympathetic ganglia.[7] Crowder[7] has suggested that these cells should be termed M1 and M3 cells, and that the M2 cells remain within the ganglia, and give rise to the sympathetic ganglion cells. The primordium of the gland is first recognized at about 33 dpc, although a difference of opinion exists as to whether there might be a mesonephric contribution that migrates into the suprarenal primordium at about this time (This possibility was proposed by Crowder[7] and denied by Jirásek.[9]) Crowder has suggested that these cells take origin from the wall of the mesonephric glomeruli. Hamilton and Mossman[13] have indicated that the sympathetic cells of neural crest origin invade the medio-dorsal region of the primitive cortical mass at about the 16-mm CR length stage (at about 44 dpc), and form a discrete group of cells in this location, although they are not completely surrounded by cortical tissue until the late fetal period. Crowder[7] has suggested that by the end of the embryonic period, the paraganglion cells (M3) now increase in number, and some of these cells appear to differentiate into chromaffin cells.

The first evidence of catecholamine synthesis is observed at about 10 weeks of gestation, although the presence of pressor amines is not observed until about the 95-mm CR length stage.[14] The principal amine in the gland is *noradrenaline* (*norepinephrine*), as the enzyme, phenylethanolamine-N-methyltransferase, responsible for its methylation to *adrenaline (epinephrine)* is not present in the medulla of the gland until after birth.[15,16]

GROSS ANATOMY OF THE POSTNATAL AND ADULT ADRENAL GLAND IN THE HUMAN

During the period between about 10 to 16 weeks of gestation, the adrenal glands have a greater volume than the kidneys. During the late prenatal period, the adrenal glands are still quite substantial structures, and at birth weigh about 0.2% of the total body weight. When their weight is compared to the total body weight, they are proportionately about 20 times greater at the time of birth compared to their proportionate weight in the adult. This is principally because the fetal cortex occupies a substantial volume of the gland at the time of birth. This region of the gland almost completely involutes within a short time after birth, and is largely replaced by adipose tissue. It has also been noted that within a few weeks after birth, the overall weight of the gland diminishes by about 50%. The gland gradually increases in weight between the second year after birth and puberty, when its maximum weight is achieved. This equally applies to the overall dimensions of the gland. While at the time of birth the adrenal is about one-third of the size of the kidney, in the adult it is closer to one thirtieth of the size of the adult kidney.

The early development of the adrenal gland is characterized by extremely rapid intrauterine growth associated with a high level of steroidogenic activity. In one study,[17] it was noted that the weight of the adrenal glands in the newborn was similar to that of the adult. However, during the first postnatal year, their weight dramatically decreased as the glands differentiated. As has been noted previously, much of the diminution in the weight of the gland that occurs shortly after birth is due to the involution of the so-called *fetal* cortex. Growth, albeit slowly, is then evident, until the age of about seven, when growth accelerates once more until puberty, when the adult weight of the gland is again achieved. Differentiation in the cells of the glomerulosa layer occurs during the postnatal period, and zonation is only completed by about the age of 12 or so, with their final differentiation into the *fasciculata* and *reticularis* zones of the gland.

The adrenal glands in the human adult are yellowish in color, and are located antero-superiorly to each kidney. They are surrounded by connective tissue that is infiltrated by a considerable amount of perinephric adipose tissue. They are enclosed within the renal fascia, but are separated from the kidneys by fibrous connective tissue. While consisting of cortical and medullary tissue,

these form embryologically as two distinct entities. In the adult, each gland weighs about 5 to 7 grams and measures about 50 mm vertically, 30 mm transversely, and about 10 mm in the antero-posterior diameter.

Sections through the adult gland reveal that its cortical region is yellow in color, and this forms the majority of its mass. The medullary region varies in color between red and grey, depending on its vascularity. The medulla forms only about 10% of the gland's adult volume. The medullary region is almost completely surrounded by the cortex, except in the region of its hilum. The thick capsule that surrounds the gland sends trabeculae into the substance of the cortex, and is also richly vascularized with arterial vessels that supply all parts of the gland.

ADRENAL CORTEX

Beneath the outer capsule of the gland, the adrenal cortex is divided into three zones, the zonae *glomerulosa*, *fasciculata*, and *reticularis*. The cell types present in these three zones vary considerably, but it is sometimes difficult to distinguish between the various zones at their boundaries.

Zona Glomerulosa

This zone constitutes about 15% of the volume of the cortex, and represents its outermost part. It is relatively poorly developed in the human compared to the situation observed in many other mammals. It consists largely of small polyhedral cells grouped either in the form of columns or as rounded masses, and these are separated by thin fibrous tissue septae that radiate in from the capsule of the gland. Their nuclei are characteristically darkly basophilic, and these cells contain only a small volume of acidophilic cytoplasm that characteristically contains small clumps of basophilic material. At the ultrastructural level, it is evident that the latter also contains numerous elongated mitochondria, many microtubules, and agranular smooth cytoplasmic reticulum characteristic of steroid synthesizing cells. While the nuclei of the more peripheral cells tend to be irregular in shape, those in the deeper part of this zone tend to be more spherical in shape.

Zona Fasciculata

This is the widest zone of the cortex, and constitutes almost 80% of its volume. This zone contains large polyhedral cells with basophilic cytoplasm arranged in long straight columns that appear to radiate out from the medulla. These columns of cells are separated by radially arranged elongated venous sinusoids. They also contain numerous lipid droplets that occupy much of the cytoplasm. These tend to be extracted during tissue preparation, and the cytoplasm therefore appears to contain numerous vacuoles. This appearance tends to be species specific, as in the human and in the rat large numbers of these lipid droplets are present within the cytoplasm, while in the hamster and in cattle far fewer are observed in this location.[18] The cytoplasm also contains large amounts of phospholipids, lipids, fatty acids, and cholesterol, all of which are embedded within a large volume of agranular smooth endoplasmic reticulum. The cytoplasm also contains large numbers of mitochondria, the ultrastructural morphology of which varies considerably between species. Because of the considerable width of this zone, in many studies this zone is subdivided into inner and outer regions.

Zona Reticularis

This zone is the narrowest region of the cortex and constitutes only about 7% of its volume. Various cell types are typically seen within the zona reticularis. In its outermost region may be found large round cells that contain much agranular smooth cytoplasmic reticulum as well as accumulations of pigmented material. Within these cells are found typically ovoid or spherical mitochondria as

well as numerous large Golgi complexes. Because of the appearance of many of the nuclei in the cells that are close to the medulla, and the limited number of organelles within their cytoplasm, the impression is formed that many of these cells are degenerating. However, little evidence of this is apparent from autoradiographic studies. Many of these cells are closely adherent to vascular capillary complexes that take origin from the elongated sinusoids that are principally located within the zona fasciculata. These vessels drain towards the capillary network within the medulla, and finally drain into the medullary veins.

GENERAL OBSERVATIONS ON THE ZONATION OF THE ADRENAL CORTEX

It has been suggested that some of the more deeply located glomerulosa cells increase in number and migrate through the zona fasciculata layer into the subjacent zona reticularis, where they may degenerate and eventually disappear. This hypothesis, however, does not appear to be confirmed by appropriate autoradiographical studies of these regions of the gland. This technique has demonstrated that most proliferative activity is seen in the zonae glomerulosa and outer reticularis regions, although no obvious *increased* ultrastructural evidence of cell death is seen in the latter region.

It has long been believed that the human adrenal gland hypertrophies during pregnancy, although this observation had initially never been confirmed histologically.[19] This is due to an increase in the degree of cellularity and width of the deepest part of the zona fasciculata.[20] In a study to investigate this point further, using histological methodology, the adrenal glands from 24 cases of sudden death during pregancy and immediately post-partum were examined. In all of these cases, the cause of death was provided, and none displayed evidence of systemic disease. The mean adrenal weight of the glands was slightly greater than in the controls (all of the controls were women of the same age who died suddenly without known antecedent illness), although the difference was not significant. However, there was a significant increase of the width of the zona fasciculata, due to an increase exclusively involving the width of the *inner* part of this zone. Reservations were, however, expressed about this finding, because of the frequent difficulty involved in measuring the width of this zone in all of the glands studied. This increase occurred early in pregnancy, and remained unchanged throughout pregnancy. It was suggested that the difference observed between the pregnant and non-pregnant state might have been related to the increased excretion of urinary cortical steroids during pregnancy. In this study, the plasma levels of 17-hydroxycorticosteroids (e.g. cortisone and hydroxycortisone) was measured at monthly intervals through normal pregnancy in 30 women, while at delivery blood was taken from the umbilical cord, and thereafter from the mother at intervals during the postpartum period. The levels rose progressively during the pregnancy, although after delivery, they returned to normal non-pregnant levels within about a week.[21]

An increase in the degree of cellularity of the deepest part of the zona fasciculata has also been noted in women of childbearing age during the summer.[22] In this study, a total of 44 women of child-bearing age who died by misadventure (In all of these cases, death was unrelated to their pregnancy. The most common causes were carbon monoxide poisoning, barbiturate poisoning, heart disease, asphyxia, subarachnoid hemorrhage, and fractured skull, but a range of other causes also accounted for their sudden deaths.), were divided into summer (21) and winter (23) groups. The widths and nuclear densities were measured in four cortical zones (glomerulosa, outer and inner fasciculata and reticularis). In the summer, the nuclear density of the zona glomerulosa was increased, although there was no increase in the width of this zone. In the outer fasciculata and reticularis zones no seasonal differences were observed. However, in the inner fasciculata, the nuclear population was increased during the summer due to a significant increase in nuclear density and width of this zone. It was suggested that the increase in the nuclear population during the summer in the zona glomerulosa might be related to an increase in sodium-retaining hormones that could account for the increase in water retention observed at that time of the year. No explanation was proposed for the increase in the nuclear population in the inner part of the zona fasciculata in the summer.

An increased degree of atrophy, associated with a significant decline in nuclear density, has been observed in each zone of the cortex of the glands of elderly males.[23] In this study, the width of the various cortical zones was measured as well as their nuclear density in 59 adult men varying in age between 20 and 86 years, all of whom had died unexpectedly, although the cause of death in each case was determined at autopsy. Care was taken to confirm that the measurements were always made at right angles to the surface of the gland. Despite the fact that coronary thrombosis was frequently associated with hypertension, a disease often associated with adrenal cortical enlargement,[24] there was no evidence of hypertensive changes observed at post-mortem examination. Similarly, there was no indication that the glands obtained from deaths due to coronary thrombosis were larger than the others. The most obvious signs of aging were observed during the 7th–9th decades. The most obvious finding was the 75% reduction in nuclear density of the inner region of the zona fasciculata, while that of the outer fasciculata was closer to 20%. The reductions in the glomerulosa and reticular zones were intermediate. Similar atrophic effects due to aging had previously been reported by others.

In a series of studies undertaken over a period of many years, Korenchevsky and others have investigated the effect of castration on female and male rats, in inducing some of the processes of aging, including those observed in the adrenals.[25-29] In females, ovariectomy hastened certain processes of aging, while the administration of exogenous hormones exerted an opposite effect on some of these processes. Similarly, castration in the male hastened the onset of age-related changes. It was believed that in both sexes, it was the decrease in the circulating levels of sex hormones that facilitated this aging effect, and that the administration of sex hormones was able to reverse some of these effects. It was suggested that the deficiency of sex hormones was not the primary cause of aging, as the changes produced were not always those observed in old animals or humans. It was, however, noted that castration performed at an early age had a more pronounced effect than late castration.

ADRENAL MEDULLA

It is often difficult to distinguish between the inner region of the cortex and the outermost part of the medulla. This is because cords of reticularis tissue penetrate a short distance into the medulla. The adrenal medulla is principally composed of chromaffin cells (or phaeochromocytes) of neural crest origin. The latter tend to be separated by wide venous sinusoids. Neural elements are also observed in this part of the adrenal gland. The chromaffin cells synthesize and release adrenaline (epinephrine) and noradrenaline (norepinephrine) into the sinusoids, and this release is under preganglionic sympathetic control.[30]

The majority of the cells of the medulla are large and columnar, and are either in rounded clusters or in short cords, being located close to the venous sinusoids, although the nuclei of these cells are distant from the sinusoids. The nerve terminals on these cells also tend to be distant from their connection with the sinusoids. The cytoplasm of these cells is basophilic, and they possess granular endoplasmic reticulum, mitochondria, Golgi complexes, and many vesicles indicative of their high metabolic activity.[30,31] The Golgi complexes contain material believed to be the precursors of the secretory granules. At the ultrastructural level, one of the most prominent features of these cells is the presence within their cytoplasm of very large numbers of small membrane-bound dense granules. In osmium stained material, the cells that stored adrenaline tended to possess pale vesicles, while in those that stored noradrenaline, the vesicles were highly electron dense. It is not entirely clear how these low molecular weight substances are retained within the granules. It has been suggested that they might form high molecular weight aggregates with ATP and divalent cations within the granules. In the human medulla, in certain age groups, both types of vesicles may be observed in the same cells.[32]

It is not entirely clear how the contents of these vesicles enter the perivascular spaces. It has been suggested, for example, that they may be released into the cytoplasm, and then diffuse through the cell membrane. Another and possibly more likely suggestion is that they are secreted by exocytosis

of the granules, and that this is stimulated by the influx of calcium ions. By one or other of these means, these substances then pass into the circulation through the fenestrated epithelium of the venous sinusoids. These then drain into the adrenal veins. While noradrenaline produces cardiac stimulation, vasoconstriction and raised blood pressure, adrenaline stimulates carbohydrate metabolism. It has been noted that the surgical removal of the adrenal medulla is not, except under certain circumstances, life-threatening, unlike the removal of the adrenal cortex.

BLOOD SUPPLY TO THE ADRENAL GLANDS IN THE HUMAN

The vascular supply to these glands is considerable, and comes from the superior, middle, and inferior suprarenal arteries. These take origin from the inferior phrenic arteries, abdominal aorta, and renal arteries, respectively.[2] All of these vessels eventually drain into the suprarenal veins. The left suprarenal vein drains to the left renal vein, while the right suprarenal vein drains to the inferior vena cava. The arteries to the gland initially tend to ramify over its surface, forming a capsular and subcapsular anastomosis or plexus, before entering the gland proper. Within the zona fasciculata the majority of the vessels are present in the form of longitudinal cortical sinusoids that are located between the cell columns in this part of the cortex. These vessels then ramify as a capillary plexus within the zona reticularis before entering the medullary region of the gland. A few of the vessels from the capsular arteries run directly to the medulla, where they then break up into capillary complexes. These vessels tend to anastomose with the capillary plexus formed by the cortical sinusoids before draining directly into the medullary veins. The medulla therefore has a dual blood supply, one component arising from the cortical sinusoids, and a second supply that arises directly from the capsular or cortical arteries. For observations on the development of the vascular supply to the adrenal gland in the rat, see later.

SOME OF THE FACTORS THAT INFLUENCE HUMAN ADRENAL GROWTH DURING THE PRENATAL PERIOD

In one study, cells from different regions of human fetal adrenal glands were isolated and maintained in tissue culture. Attempts were then made to investigate whether there was a link between the highly expressed angiotensin II receptor, type 2 (AGTR, formerly AT2) activity in the cells in the center of these glands and those that displayed characteristic apoptotic activity at about 15–20 weeks of gestation.[33] This apoptotic activity was particularly seen in the central part of the so-called *fetal* zone. It was noted that cells from the *definitive* zone of the cortex that expressed many more Ang II type 1 receptors, rather than AGTR receptors did not appear to be involved in similar apoptotic activity at that time.

In a subsequent study, these workers investigated the role of various extracellular matrix components on the fetal development of the adrenal gland.[34] Primary cultures of adrenal cells were grown on collagen IV, laminin or fibronectin, and this revealed that cell morphology and the profile of steroid secretion was affected by culture on these various matrix components. Culture on collagen IV, for example, favoured cortisol secretion after ACTH or angiotensin II stimulation, while increased dehydroxyepiandrosterone (i.e. androgen) production occurred when the AT2 receptor of angiotensin II was stimulated. Fibronectin and laminin, by contrast, decreased cell responsiveness to ACTH in relation to cortisol secretion, but increased ACTH-stimulated androgen secretion. Collagen IV and laminin increased cell proliferation, while fibronectin increased the incidence of cell death.

A recent study was undertaken to investigate the development and the functional activity of the adrenal gland during early human organogenesis.[17] This confirmed that the factors that control its growth during this period are complex. For example, it was established that during the first trimester of pregnancy, adrenal growth occurs in the absence of ACTH from the pituitary. However, after about 15 weeks of gestation, ACTH is now considered to play an essential role in allowing

normal morphological and functional development to occur, while other factors, possibly of fetal or placental origin in addition to ACTH, are also believed to be required. These include a wide range of growth factors that are believed to play a critical role in adrenal cell proliferation. Towards the end of pregnancy it has been postulated that corticotrophin-releasing hormone may also play an important role in fetal adrenal function.

STEROID PRODUCTION BY THE ADRENALS AND GONADS SHEDS LIGHT ON THEIR POSSIBLE ROLE IN THE ONSET OF PUBERTY IN MALES AND FEMALES

It appears that the different regions of the adrenal cortex produce different steroid hormones. These are mineralocorticoids (such as aldosterone and deoxycorticosterone), glucocorticoids (such as cortisol in the human or corticosterone in rodents) and some androgens (such as dehydroepiandrosterone) and estrogens, all of which are synthesized from cholesterol. Free cholesterol acts as a substrate for steroid synthesis. In the zona glomerulosa, for example, aldosterone is produced, while the cells of the zona fasciculata almost exclusively produce cortisol (in the human, but corticosterone in the mouse, see later). It is believed that the cells of the zona reticularis produce dehydroepiandrosterone. For many purposes the zonae fasciculata and reticularis appear to act as a single functional unit. What has been noted, however, is that when cortical cells are isolated from the gland, the cells from each of these zones appear to be capable of synthesizing all of these steroids, and that their output in the gland is somehow regulated by their location within the cortex.

In a study of 55 boys and 54 girls aged between 3.5 and 16.3 years, plasma levels of a wide range of sex steroids was measured by radioimmunoassay. The steroids included dehydroepiandrosterone (DHA), androstenedione (delta), testosterone (T), dihydrotestosterone (DHT), estrone (E1) and estradiol (E2).[35] The plasma levels of DHA increased in girls between 6–8 years of age, and in boys between 8–10 years of age, and another increase was observed in both sexes aged between 10–12. Delta rose in girls between 8–10 years of age, and in boys between 10–12 years of age. No increase was observed in the plasma levels of T, DHT or E1 before 12 years of age in both sexes, although E2 increased in girls between 10–12 years of age. It was proposed that the increase observed in DHA and delta, and its occurrence 1–2 years earlier in girls than in boys, suggested a possible role of these steroids in the mechanism that triggered the hypothalamo-pituitary-gonadal axis at the time of puberty.

OBSERVATIONS ON THE ADRENAL GLAND AND ITS BLOOD SUPPLY IN THE RAT

A substantial number of studies have been undertaken over the years on the rat adrenal gland, and a representative selection of these will be discussed in this section. However, for an important early study of the cellular arrangements of the rat at different ages, the reader should consult Jayne.[36] In this study, the adrenal glands of male rats aged between 17–900 days were analyzed histologically. As age advanced, the capsule increased in thickness, and became more fibrous. In all rats over 180 days, degenerating cells were found throughout all layers of the cortex, although there was considerable variability between individual rats of the same age. In general, the medulla showed less evidence of degenerative changes. Mitochondrial changes which resulted in the formation of pigmented bodies were described.

Relatively few studies, however, have investigated the development of the adrenal vascular system in the rat comparable to that of the adrenal in the human by Harrison and Hoey.[2] While not as comprehensive as the latter study, that of Tokunaga[37] is of considerable interest because of the methodology used. Microcorrosion vascular casts of the adrenal gland were studied using scanning electron microscopy. By this means it was possible to determine the sequential changes that occurred in its postnatal form. On the first postnatal day (P1), it consisted of an outer capillary layer

associated with an inner sinusoidal layer in the juxtamedullary zone. By P7, the outer capillary bed had differentiated to form the vasculature of the glomerular and fascicular zones, while the juxtamedullary sinusoidal layer differentiated to form the vasculature of the reticular zone. The medullary arteries and the capillary bed of the medulla were first seen at P14, by which time the definitive double vasculature system, as seen in the adult, had been established. It was suggested that the medullary arteries either originated from the vessels associated with the chromaffin bodies that had been incorporated into the gland, or differentiated from the radial sinusoidal vessels. For earlier comparative studies on the vascular arrangements of the adrenal gland, see Gersh and Grollman[4] and Harrison.[5]

The study by Gersh and Grollman[4] is of particular interest, because it draws attention to the fact that the number of *patent* capillaries in skeletal muscle, and presumably in other tissues, varies with its state of activity, being greater when the tissue is at work than when at rest. Similar findings have been reported for the kidney, and the same applies to the adrenal,[38] although the methodology involved in Sjöstrand's analysis has been criticized. These authors also compared the adrenal vascular pattern, and the physiological changes which it undergoes, in the adrenal glands of mice and rats of various ages and compared this with the situation in the dog. The latter species was the earliest in which a detailed vascular analysis had been carried out.[39]

The paper by Harrison[5] is extremely informative, in that it draws attention to the fact that the pattern of blood supply to the adrenals varies considerably between species. In rats and mice, the vessels involved are completely different from those that supply the adrenal in the human (see previously). Harrison was particularly interested in establishing the vascular arrangement in the rabbit, and undertook a detailed analysis of the vascular supply to the left adrenal gland in this species, as the left gland was more accessible than the right gland. He noted that in the rabbit, while the majority of the arterial blood supply came from the adrenolumbar artery, a small arterial branch was also received from the left renal artery. In the rat, as well as in the mouse, he confirmed the findings of Gersch and Grollman.[4] He noted that the blood supply to the left adrenal in the rat came principally from a relatively small vessel located some distance cranial to the origin of the coeliac artery. This small vessel then divided into two branches, one of which also provided a branch to the inferior aspect of the diaphragm. The medial part of the adrenal gland was supplied by an independent artery from the aorta which principally vascularized the under surface of the diaphragm.[5]

The blood supply to the adrenal gland in the cat differs markedly from that in the rabbit and rat, in that there are twenty or more arteries that enter the adrenal gland in the cat at all points around its periphery. These come from a variety of sources, the richest being from the renal artery. Other branches come from the adrenolumbar, coeliac, phrenic, and occasionally from the superior mesenteric artery as well as directly from the aorta. Harrison therefore confirmed the blood supply to the cat adrenal as previously described by Bennett and Kilham.[40] Harrison[5] particularly drew attention to the consequences of interrupting components of the blood supply. In the rabbit, in particular, this could lead to immediate necrosis of the zona fasciculata region of the cortex, as individual arteries to this region of the gland in the rabbit are end-arteries. The zona glomerulosa region also undergoes degenerative changes at a later period. If more than one vessel was interrupted in the rabbit, both the zona glomerulosa and fasciculata underwent immediate necrosis. Preliminary experiments also indicated that individual adrenal arteries to the cortex in the rat were also end-arteries, although the degenerative changes observed tended to be less selective in the rat than in the rabbit.

In another study, the roles of the maternal pituitary and adrenal glands in relation to the initiation of the secretion of the rat mammary glands near to term were investigated.[41] Mammary gland DNA and RNA were estimated as an indication of mammary gland development. Maternal adrenalectomy or hypophysectomy decreased the RNA:DNA ratio. However, treatment of adrenalectomized animals with cortisol acetate completely restored this ratio to the control level. Treatment of hypophysectomized animals with cortisol acetate increased this ratio, but it did not reach the control level. Removal of the pups had no effect on this ratio. Sustained maternal adrenal corticosterone production is necessary for normal mammary gland development, as shown by the lower plasma

corticosterone concentrations of adrenalectomised animals. The fetal and maternal contribution to the corticosteroids in the circulation were considered with particular respect to changes in mammary gland sensitivity to lactogenic stimulation by glucocorticoids and/or a decrease in the binding of glucocorticoids to binding protein. It was proposed that this may allow for the initiation of milk secretion just before parturition.

In a recent study, an investigation was made to determine how social relationships might influence the development of the hypothalamic-pituitary-adrenal axis in the rat.[42] In the period between P4 and P14, there is either minimal or no discernible adrenal response to stress. This is termed the stress hyporesponsive period (SHRP). Various earlier studies had indicated that maternal factors were critical for the development of this axis, and the maintenance of the SHRP. Following 24 hours of maternal deprivation, the rat displays elevated basal levels of corticosterone, and a robust response to mild stress. It was suggested that the maternal factors that regulate the diminished response of the pup to mild stress are as follows: tactile stimulation, feeding of the pups, and passive contact. These suggest that maternal behavioural factors are actively responsible for actively inhibiting the endocrine responses to stress during the early postnatal period.

Rat Adrenal Cortex

The adrenal glands in the rat are brownish in color. In the male, the total weight of the adrenals is about 30–40 mg. In the female, they are significantly heavier, with an average weight of about 47–57 mg. No enlargement of the adrenal glands was noted during pregnancy, although this has been reported in other species including the mouse. In this study, an analysis was made into the relative mass of the chromaffin tissue in the medulla as compared to the cortical tissue during pregnancy. No change in either component of the gland was observed.[43] The zonation of the adrenal cortex in the rat is similar to that in the human. It possesses a substantial zona reticularis, in addition to the zonae glomerulosa and fasciculata, although the volume of the individual zones varies considerably between individuals. What was also noted was the occasional presence of an intermediate zone between the fasciculata and the reticularis.[44]

In another early study, but along similar lines, Cain and Harrison[45] reported that considerable variations were evident in the appearance of certain regions of the adrenal cortex in the rat. They noted that the difference in appearance that they observed was principally related to the different amounts of lipids present in the cells of the zona fasciculata. They suggested that this difference in appearance might be related to a secretory phase, when large numbers of lipid droplets were present, and a discharge phase, when very few droplets were present. By contrast, they noted that in the cells of the zona glomerulosa there was almost always a considerable number of lipid droplets present, and they believed that these were probably discharged more evenly and continuously than in the fasciculata.

Relatively recent work has been undertaken to investigate the development of zonation in the rat adrenal cortex. In one study, immunochemical markers for the various zones (the zona glomerulosa, the zona fascicularis, and the zona reticularis) were used as well as markers for the medulla in order to study DNA-synthesizing cells in the adrenal glands during development.[46] Several interesting findings were reported. They noted that the separation of the cortex from the medulla and the first evidence of zonation in the cortex were observed at about the time of birth. No evidence of zonation was observed during the fetal period. Evidence of DNA-synthesizing cells was observed throughout the gland with minimal evidence of migration. DNA-synthesizing cells tended to be concentrated near to the undifferentiated zone between the glomerulosa and the fasciculata, they then migrated towards the center of the gland. Cell death was observed in the innermost region of the cortex, where many macrophages were also present. It was suggested that the undifferentiated region was likely to be acting as the stem cell zone of the cortex, and maintained cortical zonation.

A scanning electron microscopic study of the adrenal cortex has also been undertaken, and in the deeper tissues the appearance was said to be similar to that of the liver.[47] They noted that the adrenal medulla was composed of large numbers of spherical chromaffin cells, interspersed with

neural elements. For general observations on the anatomical features of the adrenal glands of the rat see Hebel and Stromberg.[48]

Rat Adrenal Medulla

Of the considerable number of studies undertaken on the adrenal medulla, some of the earlier papers described in considerable detail the morphological features observed in this region of the gland when it was examined by electron microscopy. In a subsequent series of papers by Coupland and others,[49–52] particular interest was expressed in the chromaffin cells that were involved in the development of adrenaline and noradrenaline storage in this region of the gland.

In the first of two ultrastructural studies by Coupland,[49] he reported that while the granular substance of chromaffin granules appeared to accumulate initially close to the membranes of the Golgi apparatus, it was subsequently extruded from the cell surface. Such cells often possessed cilia, and it was suggested that this might be a constant feature of these cells in the rat medulla. He was also able to differentiate between adrenaline and noradrenaline secreting cells by fixing sections initially in glutaraldehyde then transferring them to osmium tetroxide. The other cell types characteristically seen in the adrenal medulla were also detailed. In the second paper by Coupland,[50] this author described the innervation of the chromaffin cells in the adrenal medulla, particularly the terminal *boutons* previously described by Willard.[53] These consisted of synaptic-type endings on the chromaffin cells. Nerve cell bodies were also noted in the adrenal medulla, and Schwann cells normally accompanied the nerves that were identified.

In one descriptive and morphometric study, the adrenal medullas of Wistar rats aged from birth to 22 months of age were examined.[54] Primitive sympathetic cells and phaeochromoblasts were recognized by about 2 days after birth, while distinct adrenaline and noradrenaline storing cells were recognized by about 4 days after birth. Before that time, the cells appeared to contain both adrenaline and noradrenaline storing granules. While continued evidence of growth in volume of the medulla was noted throughout the entire period of the study, an increase in chromaffin cell number and volume was observed as well as an increase in the number of neural elements present. By 22 months of age, evidence of chromaffin cell hypertrophy was observed, as well as some evidence of granules that appeared capable of secreting both adrenaline and noradrenaline.

In a similar study on the pre- and postnatal development of the adrenaline and noradrenaline storing cells of rats and pigs, only the rat findings will be described here.[51] Almost identical findings to those reported above were observed. In adult animals, both adrenaline- and noradrenaline-storing cells were found, about 80% of which were involved in the storage of noradrenaline. In the earliest material studied, at about 16 days of gestation, only noradrenaline immunoreactive cells were present, while both adrenaline and noradrenaline storing cells were first noted at about 2–3 days after birth. An increase in the amount of both adrenaline and noradrenaline occurred after birth, being 13 times greater by 17 days after birth, and 100 times greater by adulthood. The relative concentration of noradrenaline present, however, steadily decreased, from 90% on the 17th day of gestation to 35% just before birth, to 20% in the adult, while that of adrenaline correspondingly increased. Three phases of development were described. First, up to the 18th day of gestation, medullary cells only synthesised and stored noradrenaline. From the 18th day of gestation to 2–3 days after birth, noradrenaline and adrenaline were synthesised and stored in a single cell type, and third, from about 2–3 days after birth, separate noradrenaline and adrenaline cell types were present. In a slightly earlier, but essentially identical study by Verhofstad and Coupland and others,[52] similar material was analysed. The adrenal medullas were obtained at daily intervals from the 16th day of gestation to the 7th postnatal day, at the 14th postnatal day, and from adults. Before birth, low but significant levels of noradrenaline were present as well as during the first 2 weeks, but in adults this accounted for less than 0.1% of total catecholamines. The other findings reported were almost identical to those reported subsequently (see above).[52]

In a series of three studies, the innervation of the adrenal medulla of the rat was studied. In the first of these studies, observations were made on the functional connections made between the

splanchnic nerve and the adrenal medulla during the pre- and early postnatal period. This study indicated that such connections do not normally become competent until the first postnatal week. This process was, however, accelerated by the neonatal administration of triiodothyronine (T3) when given daily during the first 9 days after birth.[55] This study investigated the underlying mechanism involved. It was noted that in the T3-treated animals, synaptic development was accelerated, and that there was a real increase in synaptic density in the medulla by 25 days of age. Replication of the target cells was initially enhanced, but during the long-term, however, it was suppressed, and ultimately, this led to impaired adrenomedullary function.

In a complementary study, beta-bungarotoxin (βBTX) was used to eliminate the preganglionic cholinergic nerve supply to the embryonic rat adrenal medulla in order to determine whether this detrimentally influenced the differentiation of embryonic chromaffin cells (i.e. the phaeochromoblasts). Rat embryos were injected with either 1 or 2 μg βBTX on day 17 of gestation, and isolated on day 21.[56] This injection caused a 15–20% decrease in body weight and CR length of the conceptuses and weight of the adrenals. Spinal cord development was also reduced, and acetylcholinesterase-positive cells were virtually absent from the ventral and lateral columns. The application of 2 μg of βBTX effectively reduced the neuronal input to the day 21 of pregnancy (E21) adrenal glands. However, total adrenal catecholamines and relative amounts of adrenaline and noradrenaline, tyrosine hydroxylase and phenylethanolamine N-methyltransferase activity were unaltered. Similarly, no obvious effect on the phaeochromoblasts was evident except for the lack of synapse-like axon terminals on these cells. Corticosterone levels in the adrenals and in the plasma were identical to those in controls. This study indicated that the normal development of the chromaffin cells does not require an intact nerve supply.

In a rather unusual study, embryonic and postnatal adrenal medullary cells were transferred using homologous adrenal grafts into the anterior chamber of the eye. The eyes of the recipients were adrenergically denervated 10 days prior to grafting by superior cervical sympathectomy in order to increase the levels of NGF and NGF-like activities in the iris.[57] Grafts were then taken at embryonic day 15 (E15), at the beginning of medullary progenitor cell migration into the adrenal cortical tissue, but no cortical or mature medullary cells were located after 2 weeks *in oculo*. Nervous sympathoblastic cells were, however, located on the anterior surface of the iris. By contrast, grafts transferred after the isolation of adrenal primordia at E16 and E17, and examined after 2 weeks, displayed mature cortical tissue, and these were interspersed with either phaeochromoblasts or mature chromaffin cells, despite the absence of a discrete medulla. Neural elements were also located within the cortical tissue. Sympathoblasts were located at the surface of the iris, while sympathetic nerve cells that received sympathetic axon terminals were embedded in the stroma of the iris.

When E21 cells were grafted, occasionally young chromaffin cells were located outside the cortex, and sympathetic nerve cells were often found in close contact with the cortical cells. When adult adrenal cells were transplanted, mature adrenaline and noradrenaline synthesizing cells were readily distinguished after a period of about 8 weeks even in the absence of cortical cells. These studies suggest that some degree of medullary cell differentiation is possible, although it was suggested that full transformation of mature chromaffin cells into neuronal cells was prevented from occurring in the absence of normal sympathetic neural stimulation. Large numbers of unmyelinated axons were often seen near chromaffin cells. Neuronal cells located within the stroma of the iris, however, acquire characteristic features typical of young sympathetic nerve cells. It was suggested that this might be consistent with the hypothesis that NGF produced in the sympathetically-denervated iris induced the differentiation of grafted immature adrenal medullary cells into young sympathetic neurons. Sufficient were available to allow the chromaffin cells within the graft to secrete either adrenaline or noradrenaline.

It has also been noted that while the adrenal medulla of the mouse closely resembles that of the rat, the frequency of phaeochromocytomas in the mouse suggests that it is probably a more appropriate model than the rat for studying a model system of human medullary adrenal neoplasia.[58]

DEVELOPMENT OF THE ADRENAL GLAND IN THE MOUSE

The histological features and anatomic location of the adrenal gland during the prenatal period in the mouse are illustrated in Figures 2.1–2.6.

In one of the first studies to determine the initial appearance of the adrenal medulla, Fernholm[59] investigated the timing of appearance of the sympathetic chain and its associated ganglia. She indicated that the first evidence of a sympathetic chain was observed in the thoracic region at about 11 dpc, and the first evidence of the superior cervical and stellate ganglia was recognized over the next few days. The first evidence of adrenal medullary tissue was observed on about 13 dpc with the migration of sympathoblasts into the primordia of the adrenal cortical tissue. Evidence of migration occurred over the following 3 days.

In a later detailed series of studies, the histological features of the primordium of the adrenal cortex was first seen in the mouse embryo at about 11-12 dpc as a defined region of darkly staining cells.[60] These were located just medial to the developing gonads and between them and the dorsal mesogastrium. At about the same time, medullary sympathoblasts were first evident in close proximity to the presumptive cortical tissue, and it was suggested that no further evidence of immigration of these cells was observed after about 14 dpc. No evidence of a distinction between a characteristic "fetal" and a "permanent" cortex was seen in these studies. It was only in the full-term mice that the first evidence of blood-forming cells was evident in the adrenals.

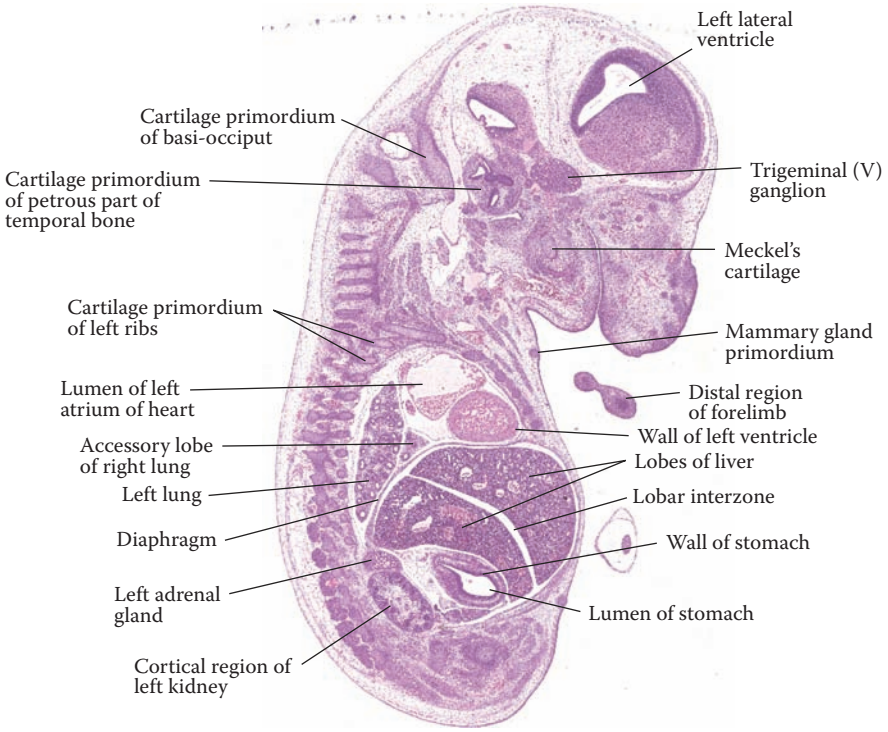

FIGURE 2.1 (E13.5_Fig2.1.svs) Low magnification, sagittal section through the left side of a mouse embryo (E13.5–14) stained with hematoxylin and eosin (H&E). Note in particular in the abdominal region the presence of the two lobes of the liver separated by the lobar interzone, a transverse section through the stomach showing the lumen of the stomach, and longitudinal sections through the left kidney and adrenal gland. The peritoneal region is separated from the pericardial cavity (superiorly) and the pleural cavity (posteriorly) by the left region of the diaphragm.

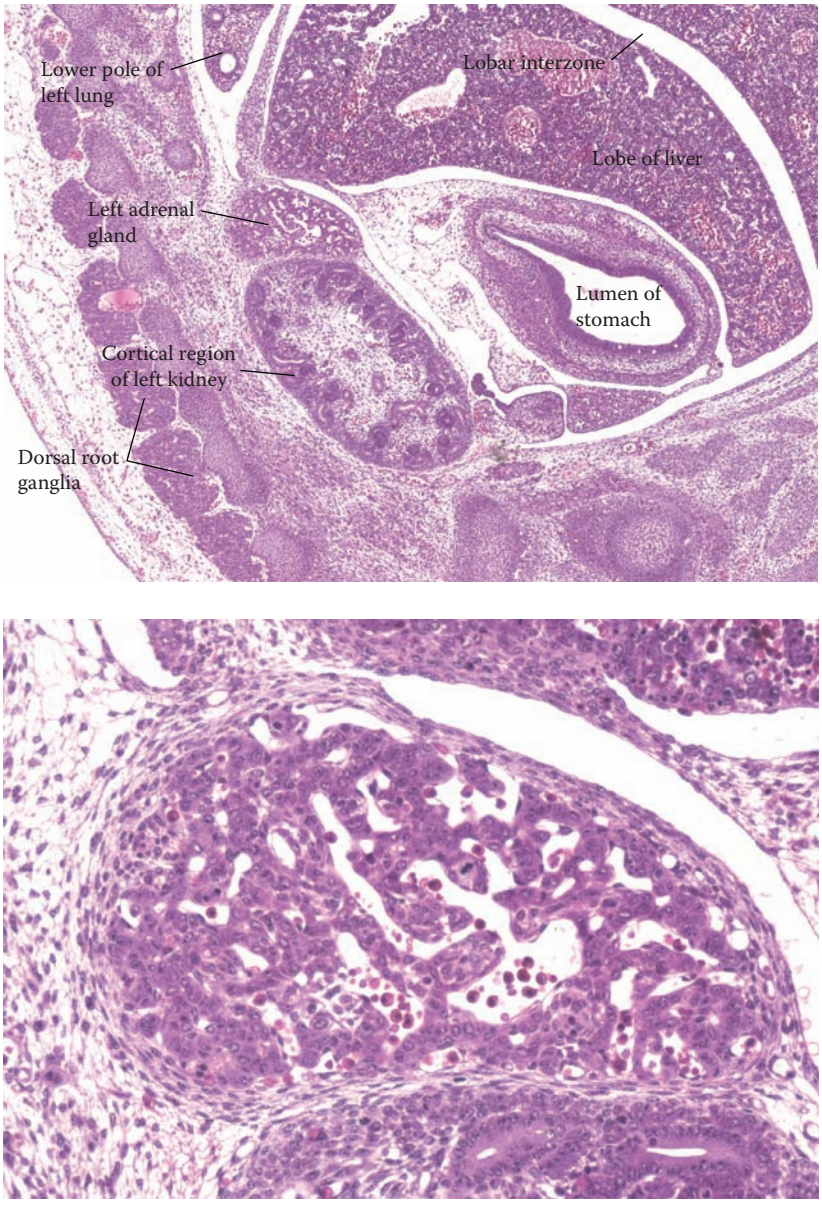

FIGURE 2.2 Medium (top) and high (bottom) magnification of Figure 2.1 illustrating the peritoneal region and its contents, but particularly the close relationship between the left adrenal gland and the left kidney.

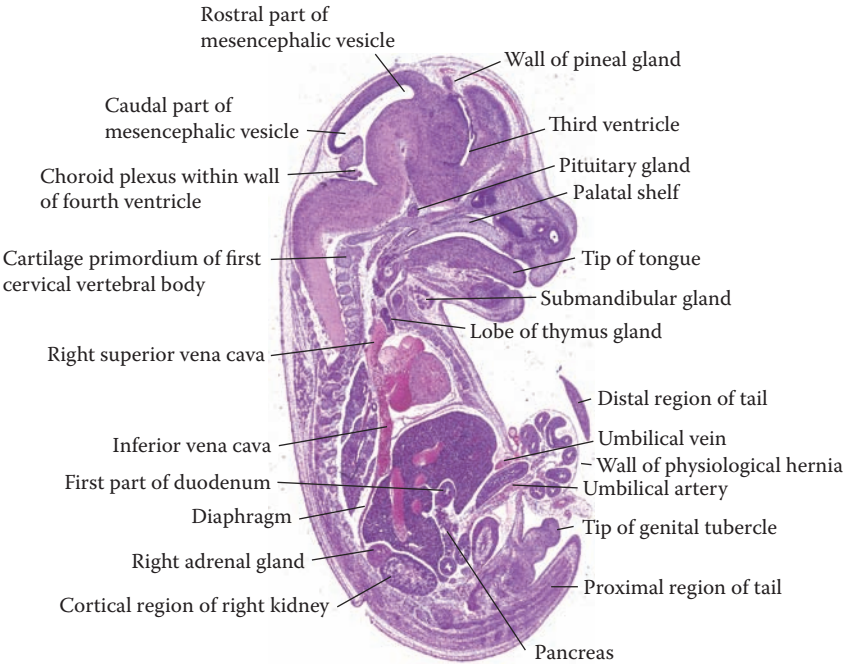

FIGURE 2.3 (E15.5_Fig2.3.svs) Low magnification, sagittal section through the right side of a mouse embryo (E15.5–16) stained with H&E. Note in particular in the abdominal region the presence of the physiological umbilical hernia, containing midgut, the genital tubercule, the front part of the duodenum, and pancreas. A longitudinal section of the right kidney and right adrenal gland are also shown. The diaphragm is seen to be pierced by the inferior vena cava.

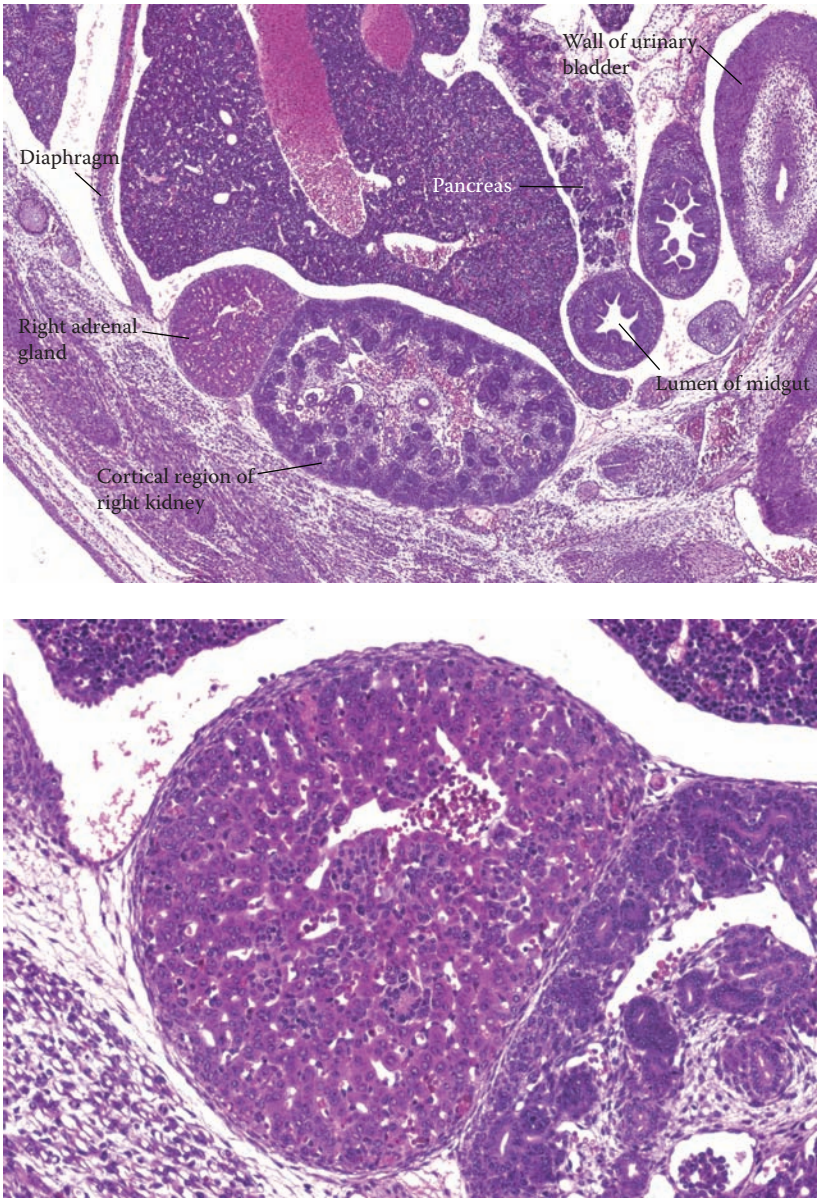

FIGURE 2.4 Medium (top) and high (bottom) magnification of Figure 2.3. Illustrates the right adrenal gland and the right kidney. The wall of the urinary bladder is also clearly seen in the medium magnification image.

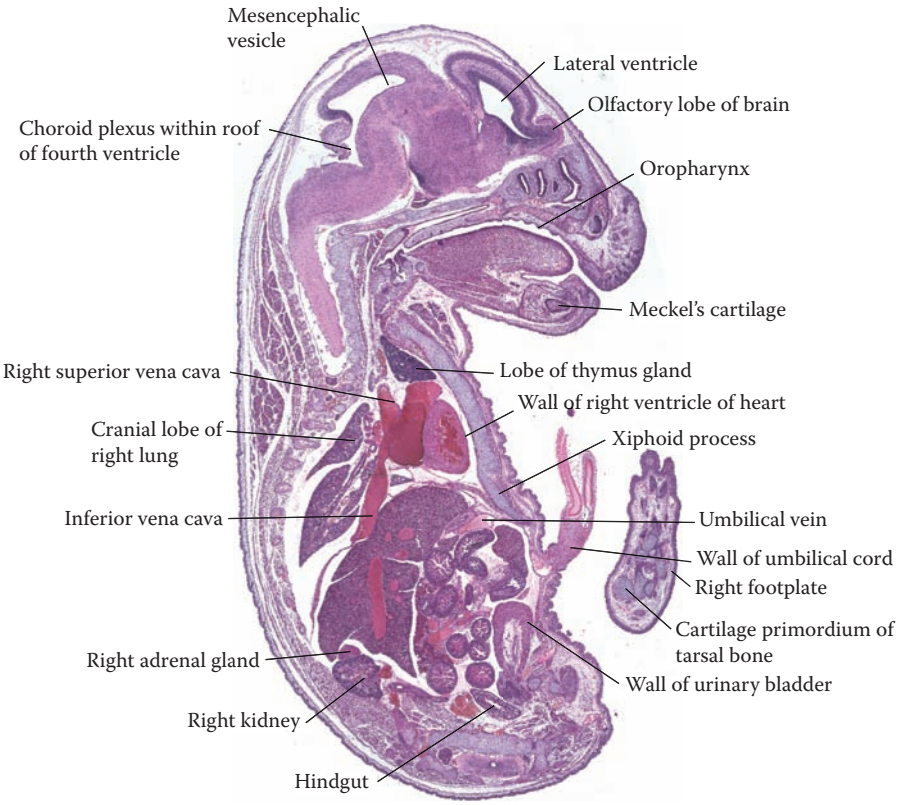

FIGURE 2.5 (E17.5_Fig2.5.svs) Low magnification, sagittal section through the right side of a mouse embryo (E17.5–18) stained with H&E. Note in particular that the physiological umbilical hernia is no longer present, and that the peritoneal cavity now principally contains the liver (rostrally) and the abdominal viscera including the urinary bladder. A longitudinal section through the right kidney and right adrenal gland are also seen.

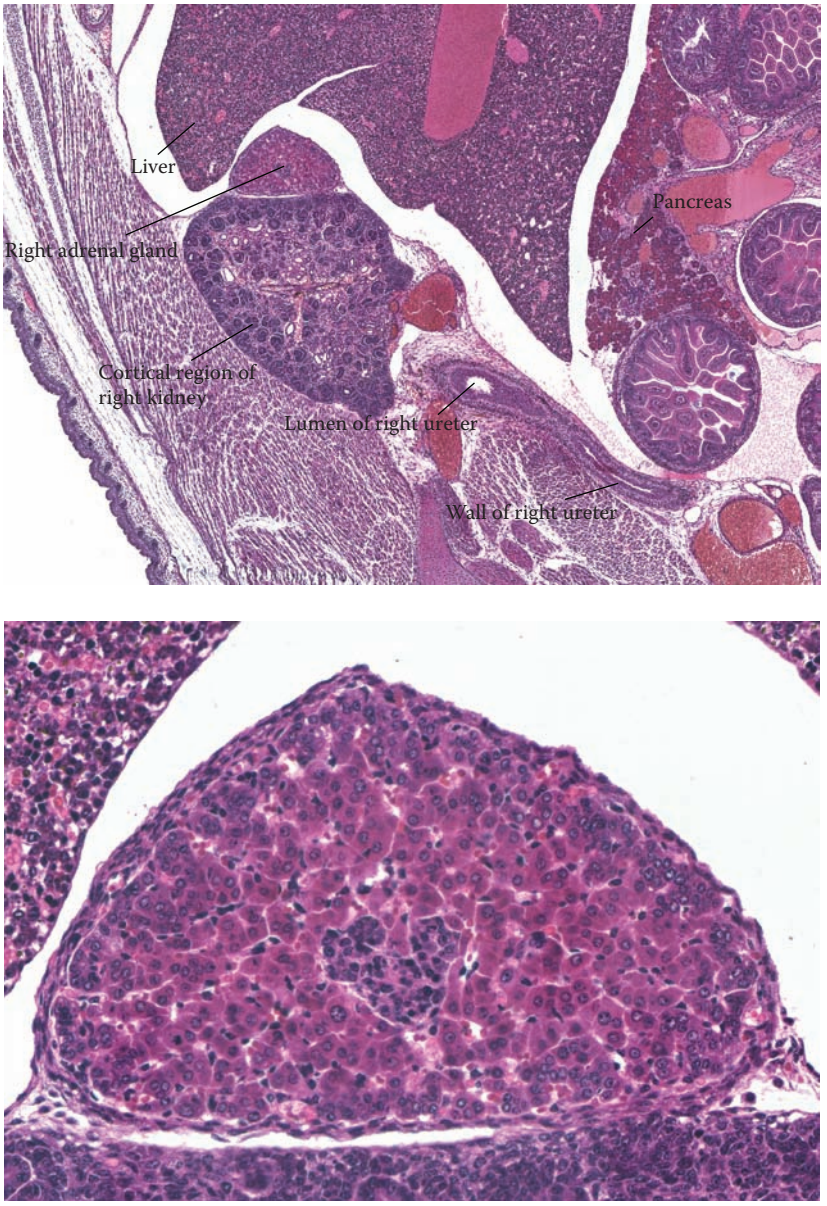

FIGURE 2.6 Medium (top) and high (bottom) magnification of Figure 2.5 illustrates the right adrenal gland and the right kidney. Note also the presence of the lumen and wall of the right ureter in the medium magnification image.

Gonocytes (primordial germ cells that are migrating from the wall of the yolk sac to the urogenital ridge that is located on the medial aspect of the mesonephros) have also been recognized within the presumptive adrenal cortical region up to about 13 dpc when the sections were stained to demonstrate the presence of alkaline phosphatase enzyme activity.[61] However, the most likely and "innocent" explanation for their presence here in isolated sections through this region at this time is that this is along the pathway of migration of these cells from the dorsal mesentery of the hindgut to the presumptive gonads.[62]

ANATOMICAL FEATURES OF THE ADRENAL GLAND IN THE MOUSE

These glands are located above the antero-rostral poles of the kidneys, the right adrenal gland being closer to the kidney than the left,[63] and the venous drainage differs between the two sides. Strain differences have also been described. The overall weight and appearance of the gland differs between the male and the female, with the left gland typically weighing more than the right gland.[64] The weight in the female is about 25% greater than in the male, while the gland is said to be more opaque in the female. This is due to the presence of an increased amount of lipid in it.[65] In the mouse, the adrenal cortex comprises about 80% of the total volume of the gland, while the medulla occupies about 20% of the gland.

If present, accessory medullary tissue, consisting almost exclusively of chromaffin tissue, is particularly commonly seen near to the left renal vein. An early description of a number of these structures, referred to them as discrete para-aortic chromaffin "bodies" that were located in the pre-aortic region adjacent to the renal vessels.[66] These "bodies" were reported in both the mouse and guinea pig. In the young mouse, it was noted that such a "body" disintegrated. While some of its constituent phaeochromocytes degenerated, others persisted to form small, localized masses in the pre-aortic region. It was suggested that the differential growth of sympathetic nerve fibers, and the presence of other chromaffin elements stimulated their presence. It was also noted that small non-encapsulated collections of chromaffin cells were occasionally found in the ganglia of the lumbar para-vertebral sympathetic chain.

The extra-adrenal chromaffin cells were first described by Zuckerkandl[67] in the abdominal sympathetic plexuses of the human fetus, and were principally located close to the region of the inferior mesenteric artery. Such structures are also observed in other mammals, and their post-natal fate has also been described.[14] The pressor agent present in the human newborn is predominantly noradrenaline.[15] Accessory cortical nodules are also relatively commonly encountered in the adult.[68,69] According to Gruenwald, accessory adrenal cortical nodules located near the adrenal capsule are a relatively common occurrence in human infants and young children. These are believed to initially grow by apposition to the main gland, and that many of these accessory nodules eventually fuse with the main gland.[70] In the mouse, discrete nodules of adrenal cortical tissue were found in close proximity to the adrenals and kidneys in a considerable number of strains of mice.[68] Such nodules were found in about half of the mice examined, although in a far lower proportion in hybrid strains. Such nodules occurred either singly or in small groups, and were usually located very close to blood vessels. Most were unilateral and on the left side, while some were bilateral or on the right side. The histological morphology of these nodules revealed that they tended to be composed of the layers typically seen in the normal adrenal cortex. Aging changes in these nodules were also similar to those observed in normal glands. It is of importance to be aware of the existence of accessory nodules of adrenal tissue, particularly in experiments in which complete adrenalectomy is required. If this is the case, then every effort must be made to locate and remove these islets of tissue. Information is available on searching for these islets and their removal.[71,72] While it is possible to recognize both the zonae glomerulosa and fasciculata in the adult mouse, these zones are not well differentiated and tend to merge into each other.[73,74] The extensive review by Zelander[73] is now of limited value, because the cortex of the mouse is divided into four zones. These are termed the glomerulosa, inner and outer fascicular,

and reticular zones. The presence of the latter zone is now disputed, and unlike the situation commonly observed in other mammals, no zona reticularis is considered to be present in the mouse adrenal cortex. For observations on the functional changes that occur in the adrenal cortex associated with aging, see Yarrington.[75]

HISTOLOGICAL MORPHOLOGY OF THE ADRENAL GLAND IN THE MOUSE

Zona Glomerulosa

The cells in this part of the cortex are small, and this region of the cortex is relatively narrow and located just beneath the capsule of the gland. The cytoplasm of these cells is basophilic. Their mitochondria tend to be round or ovoid in shape, and possess tubular cristae. They also possess round perinuclear lipid droplets of uniform size.[76]

Zona Fasciculata

The cells in this region of the adrenal cortex are arranged in columns, while their cytoplasm is eosinophilic. These cells also possess large numbers of lipid droplets within their cytoplasm and round mitochondria.

It has also been noted that considerable numbers of cells that store vitamin A are scattered throughout this zone.[77] In Hirosawa and Yamada's study,[77] they noted that vitamin A was stored within lipid droplets in the cytoplasm of fibroblast-like cells that were mostly distributed in the region between the capsule and the medulla. The highest incidence of these cells, however, was located in the adrenal cortex, principally in the zona fasciculata, whereas only relatively low levels were seen in the zona glomerulosa. In the medulla, vitamin A containing cells were less frequently found than in the cortex, although at the cortico-medullary junction the lipid droplets tended to be larger than elsewhere.[78] Popper and Greenberg[78] also found labeled cells in the epithelial and Kupffer cells of the liver, as well as in the fascicular layer of the adrenal cortex, in the interstitium of the kidney and lung and in numerous other sites. They suggested that carotene was probably converted into vitamin A in one or all of these locations.

As indicated above, such vitamin A storing cells are also found in other organs in the mouse. They also noted that the organ with the highest concentration of this vitamin was the liver, and that it was located in the cytoplasm of fat-storing cells in this organ.[79] In a preliminary report, Hirosawa and Yamada[80] confirmed the findings of Popper and Greenberg.[78] They observed that radioautographic labeling of vitamin A storing cells revealed that this vitamin was concentrated in the lipid droplets of fat-storing cells in numerous locations. These included the perisinusoidal space in the liver, in the alimentary canal from the esophagus to the large intestine, and in cells of the lamina propria of the intestine. In the adrenal, it was concentrated in cells between the parenchymal and endothelial cells. Labeled cells were also seen in the alveolar septa in the lung. While their role is obscure, it has been suggested that because they are located in organs where vitamin A metabolism is high, they may play a role in the metabolism and storage of this vitamin.

The X Zone of the Cortex

The terminology of the various regions of the adrenal cortex in the mouse is dissimilar to that described in the human and in the rat. The *X-zone* (androgenic) of the mouse adrenal cortex was first described by Masui and Tamura in 1926,[81] and many of its features confirmed by Howard-Miller in 1927[83] and later by Hirokawa and Ishikawa.[82] According to Masui and Tamura,[81] this zone is first evident in the form of a small group of eosinophilic cells located in the innermost part of the adrenal cortex from about 10–14 dpc. In the male mouse, this zone is said to soon

cease growing, and entirely disappears by the time of puberty. In the female, this zone continues to grow until 4–5 weeks after birth, by which time it occupies a large proportion of the total volume of the gland. This zone is eventually replaced by fatty infiltration. In the virgin female it may remain until the female is three months of age, while in the pregnant female it rapidly involutes. Howard-Miller[83] first recognized the X-zone in the mouse at about 3 weeks of age, and its degeneration in male mice was noted at 5 weeks of age. The timing of its degeneration in female mice was also confirmed. Attention was also drawn to the similarity between this zone in the mouse and the so-called fetal zone in the human. It has also been suggested that the X-zone in the mouse is identical in function with the more peripheral fascicular and glomerular zones, although it differs from these other zones in that it is less responsive to stimulation, and is more readily depressed, reacting to only extreme degrees of stimulation.[84] In contrast to the findings of Masui and Tamura,[81] Hirokawa and Ishikawa[82] noted that the first evidence that they observed of the X-zone in the mouse was seen in the inner region of the cortex at about 8 days after birth. They observed small groups of cells in the juxtamedullary region that differed morphologically from the cells in other parts of the inner region of the cortex. At the ultrastructural level, these cells contained nuclei with an irregular outline and also contained in their cytoplasm parallel stacks of flattened smooth endoplasmic reticulum. Several days later a narrow layer of small eosinophilic cells was identified in the adrenals of both sexes. These cells were readily recognized at the light microscopic level. On ultrastructural examination, these cells displayed all of the characteristic features of cells from the X-zone, with many mitoses and bizarre mitochondria. In the males, they reported that these cells often involuted at about 30 days, whereas in females they persisted for a considerably longer period, until either pregnancy intervened or the earliest signs of fatty degeneration were apparent.

In the study by McPhail and Read,[85] into the possible origin of the X-zone in the mouse, they indicated that this zone was capable of regeneration following pregnancy and after the administration of testosterone propionate. They suggested that the new X-zone that formed was probably derived from the zona fasciculata.

MORPHOLOGY AND FUNCTIONAL ACTIVITY OF THE ADRENAL MEDULLA IN THE MOUSE

The adrenal medulla in the mouse comprises about 20% of the total volume of the adrenal gland. It is located in the most central part of the gland, and is completely surrounded, except at its hilar region, by adrenal cortical tissue. It tends to be reddish in color, and is largely composed of chromaffin cells. These are involved in the storage and secretion of the catecholamines adrenaline and noradrenaline. Other cells present in the medulla consist of sympathetic ganglion cells, venules, and capillaries. This region of the gland is also morphologically similar in both sexes. The cytoplasm of the medullary cells is granular and more basophilic than that of the cortical cells, and contains large numbers of chromaffin-positive secretory granules. Two types of chromaffin cells have been recognized in the adrenal medulla, largely based on their staining characteristics, and are said to be related to the two catecholamines secreted by this region of the gland.[86]

In the adult mouse, a high proportion of these cells appears to be adrenaline secreting, while only about one third to one quarter are noradrenaline secreting.[87] The latter are principally located in the region of the medulla closest to the adrenal cortex. The density of the two types of granules within the chromaffin cells differs, so that those that are involved in the storage of adrenaline are moderately dense, while those that store noradrenaline are extremely dense. Sympathetic ganglionic cells are distributed throughout the medulla.

It has now been established that acetylcholine is the immediate physiological stimulus that causes the chromaffin cells to release their catecholamine hormones, by acting on the outer surface of these cells,[88] possibly by promoting the inward movement of calcium across the cell membrane.

The catecholamines are usually present in high levels in the medullary tissues. In the human, it has been noted that with increasing age, the proportion of adrenaline present increases.[15,89]

The release of one or more hormones from the medulla appears to depend on the nature of the activating stimulus. Thus, for example, hypoglycemia and asphyxia principally causes the release of adrenaline, while an increase in the secretion of noradrenaline results from carotid occlusion. It has been suggested that the overall control of the secretion of these two catecholamines is probably under different cerebral cortical control, and that this results in the stimulation of different regions of the hypothalamus. Lewis[89] details examples of the considerable range of different types of stimuli that selectively induce the stimulation of one or other of these hormones. It has recently been suggested that the central control of this system is from the medulla oblongata and pons, as well as from the hypothalamus. It has also been suggested that the sympathetic innervation of the adrenal medulla might be under the direct control of the thoraco-lumbar spinal cord from which the preganglionic nerve fibers innervate these chromaffin cells.[90]

FUNCTIONAL ACTIVITY OF THE DIFFERENT REGIONS OF THE ADRENAL CORTEX IN THE MOUSE

While, as indicated previously, the adrenal cortex comprises the majority of the adrenal gland, its regulation is completely independent of that of the medulla (see above). As also noted previously, the adrenal cortex is considered necessary for life as its hormonal output plays a critical role in the regulation of essential somatic processes. The medulla, by contrast, while an important extension of the sympathetic system, is not considered essential for life, as long as the animal is not significantly stressed.

At the ultrastructural level, the steroid secreting cells of the adrenal cortex have certain features in common with cells of other steroid-secreting glands elsewhere in the body. All possess an abundance of smooth endoplasmic reticulum associated with a relative lack of rough endoplasmic reticulum. They possess abundant round mitochondria that have certain structural features in common, but differ from those found in the cytoplasm of non steroid-secreting cells.[91] They also tend to possess many large lipid droplets and septate-like cell contacts.[92]

The mitochondrial morphology of the steroid-secreting cells differs from that of mitochondria in other cellular systems where characteristic cristae with shelf-like membranous infoldings are present. The majority of those in the steroid-secreting cells possess characteristic tubular finger-like invaginations. Differences in mitochondrial morphology are even observed in the different zones of the adrenal cortex. Thus in the zona glomerulosa, they tend to possess lamellar or tubular membranes, while in the zona fasciculata, they tend to be more vesicular in appearance. It has also been noted that these differences tend to be species specific.[91]

The hormonal output of the adrenal cortex may be divided into three distinct groups. These are the mineralocorticoids, the glucocorticoids, and the sex hormones (in the human and in many other mammalian species, but probably not in the adult mouse). While the cortex is principally regulated by a feedback loop involving the hypothalamic-anterior pituitary axis, other feedback loops have also been described. The principal hormonal output of the zona glomerulosa is aldosterone, one of the mineralocorticoids. Its principal role is in the retention of extracellular fluid electrolytes, particularly sodium and potassium. Should there be an inadequate secretion of this hormone, the fluid volume decreases, and death due to hypovolemic shock results. The circulating levels of sodium and potassium are regulated within very narrow limits by the renin-angiotensin system. It now also appears to be accepted that the cells of the zona glomerulosa function independently of anterior pituitary control.[93]

The principal hormones secreted by the zona fasciculata in the human and guinea pig are the glucocorticoids, such as cortisol. These hormones influence the circulating level of glucose within the blood plasma. It is thus involved in glucose utilization. In the mouse and rat, the principal

glucocorticoid secreted is, however, corticosterone, and its synthesis and release is controlled by a feedback loop involving the secretion from the anterior pituitary gland of ACTH.

It has been suggested that progesterone is the precursor of the corticosteroids. One suggestion is that acetate ions are built up into cholesterol with the intervention of coenzyme A in the smooth endoplasmic reticulum. The cholesterol side chain is then cleaved off in the mitochondria with the synthesis of pregnenolone. Progesterone is then formed from this in the smooth endoplasmic reticulum, and then deoxycorticosterone by its hydroxylation. In the mitochondria this is converted to corticosterone, cortisol, and aldosterone.[91]

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3 The Pituitary Gland

INTRODUCTION

In any consideration of the endocrine system, it is necessary to draw attention not only to the overall control of this system but also to how the various organs that together constitute this system interact to control homeostasis. Possibly of greatest importance involves the control of anterior pituitary hormone secretion by the hypothalamus. General observations on the clinical relationship between these two structures have long been known, and examples are provided, where appropriate, throughout the text. As the terminology used for describing the development and various components of the pituitary gland often depends on the species studied and source of information, it was considered useful to provide information on this topic at an early stage in this chapter.[1–7]

It is now also appreciated that the hypothalamus controls the anterior pituitary gland via the pituitary portal venous system. By this means, the median eminence of the hypothalamus is now known to be closely associated with the anterior part of the pituitary gland. As a result of numerous largely experimental studies, it now appears clear that the hypothalamus controls the anterior pituitary indirectly via chemotransmitters that are mediated through the hypothalamo-pituitary portal venous system. Of critical importance would appear to be an understanding of the role of certain releasing factors produced by the hypothalamus and how they act via the pituitary gland. By this means, the control of a wide range of bodily functions is achieved. For example, these releasing factors are now known to play a critical role in the control of reproduction, growth, and development as well as in the maintenance of fluid balance and the control of the body's response to stress. These functions are all achieved through the control of pituitary hormone production by the hypothalamus. For observations on the interrelationship between the hypothalamus and pituitary and how neural control of glandular secretion is achieved, see Cone et al.[2,8]

The intimate relationship that is established between the hypothalamus and pituitary gland occurs at a very early stage of mammalian development, and how this arises in the human, the mouse, and to a lesser extent in the rat is considered in this chapter.

GENERAL OBSERVATIONS

Most of the information on the early stages of development of the organs that constitute the endocrine system was first described in the human embryo and fetus. To allow a species comparison to be made with the human, information is first provided on the degree of development achieved in the pituitary gland of the human embryo[9] and fetus[3] during all stages of its organogenesis. Information from the analysis of appropriately stained human sectioned embryonic material also allows the acidophilic and basophilic cell types present at specific stages of development to be recognized. Specific immunoreactive techniques applied to more advanced stages of development now allow the various cell types present at these later stages of gestation to be distinguished and the various substances that they produce to be recognized. After this information has been considered, it is then appropriate to discuss the comparable information available on the pre- and post-natal development

observed in rodents. As a considerable volume of complementary information was obtained from rodent studies over the years, this is accordingly described next.

TERMINOLOGY

One of the potential problems encountered by those reading about the pituitary gland (or hypophysis cerebri) is that the terminology used can vary quite considerably (see above). The standard terminology used is as follows. The pituitary gland is derived from two sources: (1) Rathke's pouch (RP); and (2) the part of the pituitary gland that is derived from the floor of the third ventricle. The Rathke's pouch is derived from oral ectoderm and gives rise to the adenohypophysis—this includes the *pars anterior* (or pars distalis or pars glandularis or anterior lobe), the *pars intermedia* (intermediate lobe), and the *pars tuberalis* (this part of the anteror lobe extends upward on either side of the pituitary stalk and may sometimes completely surround it).

The part of the pituitary gland that is derived from the floor of the third ventricle (or diencephalon) is termed the *neurohypophysis* and is derived from neuro-ectoderm (or neural ectoderm). This includes the following parts of the definitive gland: the *pars posterior* (or posterior or neural lobe), the *infundibulum* (or infundibular stem or pituitary stalk), and the *median eminence*.

The component cells and the hormones they produce that are located in the various parts of the gland are as follows:[6]

A. *Posterior lobe* (pars nervosa): Magnocellular neuron terminals (oxytocin, vasopressin).
B. *Intermediate lobe*: Melanotropes (melanocyte-stimulating hormone, MSH) and α- and γ-endorphins.
C. *Anterior lobe* (pars distalis, pars tuberalis):
    1. Corticotrope (adrenocorticotrophic hormone, ACTH).
    2. Thyrotrope (thyroid-stimulating hormone, TSH).
    3. Somatotrope (growth hormone, GH).
    4. Lactotrope (prolactin, PRL).
    5. Gonadotrope (luteinizing hormone, LH; follicle-stimulating hormone, FSH).

The terminology indicated differs from that in *Gray's Anatomy*.[1] The terminology provided there is as follows:

Anterior lobe (pars anterior): Chromophil cells, chromophobe cells, and folliculostellate (FS) cells.

A. Chromophil cells.
    1. Acidophils (α-cells).
        a. Somatotrophs (somatotropin, GH).
        b. Mammotrophs (PRL).
        c. Mammosomatotrophs (GH, PRL).
    2. Basophils (β-cells).
        a. Corticotrophs (ACTH).
        b. Thyrotrophs (β-basophils, TSH).
        c. Gonadotrophs (δ-basophils, FSH, LH).
B. Chromophobe cells.
C. Folliculostellate (FS) cells.

It should be noted that the terminology used in some of the earlier studies may also differ from that described here.

INFORMATION ON THE EARLY DEVELOPMENT OF THE PITUITARY GLAND IN THE HUMAN EMBRYO AND FETUS[10]

In the early human embryo, even before the neural folds close in the region of the anterior (or rostral) neuropore,[9,11] a small ectodermal placode forms an evagination, and this appears in the roof of the stomodeum (the stomatodeum, buccal, or oral cavity) and is destined to give rise to Rathke's pouch (or hypophyseal recess). Shortly afterward, this is seen as a midline indentation in the roof of the buccal cavity. It is located just in front of the buccopharyngeal (or oropharyngeal) membrane and close to the tip of the notochord. It is, at the same time, also located close to the floor of the diencephalic region of the future third ventricle of the neural plate. Shortly after the rupture of the buccopharyngeal membrane, Rathke's pouch is located just in front of the junction between the *ectoderm* of the buccal cavity and the *endoderm* at the rostral extremity of the pharyngeal region of the foregut. Over a relatively short period of time, Rathke's pouch becomes increasingly indented into this part of the roof of the oropharynx, while the exact location of the junctional zone between the ectoderm of the roof of the oropharynx and the foregut endoderm soon becomes impossible to discern.

Shortly afterward, the neck of Rathke's pouch becomes constricted at its site of attachment to the roof of the oropharynx. It is also at about this time that the roof, or dorsal extremity, of Rathke's pouch makes contact with the infundibulum. This is the situation emphasized by O'Rahilly.[12] He noted that at Carnegie Stage (CS) 13, the basement membrane of the craniopharyngeal invagination and that of the brain are clearly in contact, from which it could be implied that *before* this stage this was not the case. He later restated this view in the following terms: "The overlying neuro-ectoderm is the infundibular area [i.e., the pituitary stalk] and the hypophysis [i.e., the anterior pituitary] arises at a single locus, not from two distinct evaginations that approach each other and fuse."[13] This view would appear to be consistent with the situation observed at a comparable stage of development in the mouse, from the analysis of conventional histological sections through this area.[14]

However, it was stated in 1993 that from their *ultrastructural* analysis of human embryos aged between 3 and 5 weeks of gestation, there was "no direct contact with the diencephalon."[15] Stefanovic et al. continued, "However, even at these contact sites [i.e., between the primordia of the anterior pituitary and that of the posterior pituitary and pituitary stalk] a thin layer of extracellular material always remained between the tissues, elsewhere covered by a well-developed basement membrane. RP [i.e., Rathke's pouch] was, in addition**,** surrounded by close-coming mesenchymal cells during the time studied." This observation would appear to argue against O'Rahilly's inductive interaction hypothesis.[12] A similar suggestion to that of Stefanovic et al. had also previously been proposed by Ikeda et al.[16] In this paper, the authors suggested that before 5 weeks of gestation, RP stretched rostrally from the stomodeal epithelium to the middle of the mesoderm. Only after this period was it "involved with the diencephalon." It was only "after 13 weeks of development [that the two parts of the pituitary gland] had a position similar to that found in the newborn infant." This article continued, "The closer their relationship was to the diencephalon, the greater were their epithelial characteristics."

Both the infundibulum and the posterior pituitary are ectodermal (technically neuro-ectodermal) in origin and develop from a downgrowth of the floor of the diencephalon at this site. Shortly afterward, RP normally loses its contact with the roof of the oropharynx when its increasingly narrow connection between it and the roof of the oropharynx is severed. Later in this chapter, observations on when this event does not occur are provided. However, before complete separation occurs and RP loses its connection with the roof of the oropharynx, it remains connected to it by a solid cord of cells. The walls of RP are destined to give rise to the various parts of the anterior pituitary (or adenohypophysis), while the infundibulum extends caudally and ventrally to form the posterior lobe of the pituitary gland (or neurohypophysis). The narrow connection between the floor of the diencephalon and the posterior pituitary loses its lumen and is then termed the infundibular (or pituitary) stalk. The largest part that is derived from RP is the pars anterior (or pars distalis), from the upper and lateral parts of which areas extend initially on either side of the infundibular

stalk, and may sometimes completely surround it. These wing-like extensions of the pars anterior are also termed the pars tuberalis. The part of the anterior pituitary that makes direct contact with the neuroectodermally derived pars nervosa (or posterior pituitary) is separated from the rest of the pars anterior by a narrow cavity. This represents the remnant of the original lumen of RP. In most species, except in the mouse (see below), this part of the pars anterior is usually relatively thin and is termed the pars intermedia.[10]

During the differentiation of the pars anterior, this region of the pituitary becomes richly vascularized, and the cells from this part of the gland form a meshwork around these blood vessels. Relatively recent studies suggest that the different cell types located here form in succession.[17] A well-defined portal venous system is recognized during the early fetal period. Some of the pars anterior cells accumulate acidophilic granules at the beginning of the third month of gestation,[18] and shortly afterward, at about 3.5 to 4 months of gestation, basophilic granules are first seen in other cells. Most of its cells, however, remain chromophobic throughout fetal life, as do those of the pars intermedia and pars tuberalis. By contrast, most of the cells of the pars nervosa become modified to form the pituicytes. These appear to largely consist of modified astrocytes.[19] Relatively few of the cells in this part of the gland differentiate to form nerve cells, although nerve fibers grow into it from the hypothalamic nuclei via the infundibular stalk. Neurosecretory activity is seen in certain parts of the hypothalamus, and nerve fibers pass from these regions into the pars nervosa during the late fetal period.

It is relevant to note that the timing of the earliest events associated with the development of the various components that give rise to the pituitary gland in the human varies considerably between the different sources. While the descriptive account above is reasonably comprehensive, the timing of first appearance of the various structures is somewhat vague. The study on specific embryos by O'Rahilly and Müller[9] is the source for the following list, which is supplemented by the more detailed observations of O'Rahilly.[12,13]

CS 10,[9] at approximately 22 days post coitum (dpc),[9,20] "area of future neurohypophysis approximately delineated (future site of neurohypophysis and mamillary region)." This represents the stomatodeal-derived ectodermal craniopharyngeal invagination and is located immediately rostral to the oropharyngeal membrane.[21] The region from which the neuroectodermally derived part of the pituitary, the pituitary stalk, and the posterior pituitary (or neurohypophysis) is formed is from the infundibular region of the floor of the third ventricle. These are believed to form at about the same time. They approach each other and subsequently fuse.

CS 11,[22–25] at approximately 24 dpc, "… first evidence of primordium of adenohypophysis. Ectodermal region of summit of oropharyngeal membrane." Note that the closure of the rostral neuropore also occurs during this stage.

CS 14, at approximately 32 dpc. At this stage, RP is now readily seen, and the notochord appears to be inserted into its dorsal wall. As the pouch elongates, blood vessels invade the region between the basement membranes of the pouch and brain.

CS 16, at approximately 37 dpc. An evagination in the floor of the third ventricle associated with the first evidence of the neurohypophysis is seen in only about 50% of embryos studied but is not very distinct. It was, however, distinct in all embryos at CS 17 (at approximately 41 dpc). The adenohypophysis was first noted at this stage. It was described as being "… still open towards the pharyngeal cavity."[18]

CS 19, at approximately 47.5 dpc, said to be "first evidence of the pars intermedia as well as the pars tuberalis." The infundibular stalk was "relatively thick" and "contained remnants of the lumen of the hypophyseal (sometimes spelled hypophysial) sac."

CS 20, at approximately 50.5 dpc, "… stalk long and slender, capillaries appearing in the mesoderm at the rostral surface of the adenohypo*physis*."

CS 21, at approximately 52 dpc, "… thread-like stalk beginning to be absorbed" and becomes fragmented.

CS 22, at approximately 54 dpc, "remnants of the incomplete stalk present at each end."
CS 23, at approximately 56.5 dpc, "… stalk no longer seen; lobules of epithelium project into mesodermal component of gland; oriented epithelial follicles appear; abundant angioblasts and capillaries found."[18,26]

PITUITARY GLAND IN GENETICALLY ABNORMAL HUMAN CONCEPTUSES

A small number of studies were carried out to investigate the morphological appearance of the pituitary gland and the *sella turcica* (i.e., the transverse depression on the dorsal/upper surface of the sphenoid bone in which the pituitary gland is located) in either genetically abnormal human conceptuses (e.g., with trisomy 21) or morphologically abnormal conceptuses (e.g., with anencephalus).

In the first of these studies, the findings in the pituitary region were related to the ossification pattern of the axial skeleton that formed the cranial base and upper cervical or the sacral regions of the vertebral axis.[27] A total of 22 fetuses between 14 and 21 weeks of gestation were examined, and their features were compared with age-matched normal fetuses. Four types of pituitary/sella turcica morphology were described. A total of 13 Type I fetuses were morphologically normal. In six fetuses minor abnormalities were observed in the region of the sella and pituitary, and evidence of a pharyngeal pituitary was observed in some of these cases (termed Types II and III, respectively). In three cases, more pronounced abnormalities of the sella were described. For example, radiological examination of the region of the sella indicated that the basisphenoid region appeared to be cleft in the region anterior to the sella.[28] In all these cases, the basilar part of the occipital bone appeared to be normal. These minor radiological abnormalities were often associated with cervical vertebral abnormalities. In the most severe cases, these were also associated with malformations of the lumbar vertebrae.

In two papers, the influence of the hypothalamus on the development of the cells of the anterior pituitary were studied in anencephalic and in matched normal human fetuses. In the first of these papers, an immunocytochemical method was used to study the size of the pituitary cells during early gestation.[29] It was noted that the somatotrophic and corticotrophic cells were present by the end of the second month of gestation. However, after the third month the continued differentiation of these cells did not appear to persist in the absence of the overlying hypothalamic region of the brain, as was commonly observed in the anencephalic fetuses.

In a second paper in which similar material was analyzed,[30] immunohistochemistry was used to localize the presence of various hormones within the anterior pituitary. The only obvious difference observed was a decrease in the ACTH-producing cells in the anencephalic embryos. It was noted that this was the earliest hormone recognized in the normal embryos and was observed at about 5 weeks of gestation. All the other hormones were present at about 13 weeks of gestation. It was noted that the atrophy of the adrenal cortex that had previously frequently been reported in anencephalic fetuses was probably correlated with the decreased hormone production by the pituitary noted in these embryos. This appeared to be reflected in the absence of alkaline phosphatase activity in the adrenal cortex in anencephalics. The component of the adrenal cortex that is poorly developed in anencephalics is the transient *fetal* cortex.[31]

GROSS ANATOMY OF THE PITUITARY GLAND IN THE HUMAN ADULT

Any differences between the pituitary in the human and the mouse clearly relate to the considerably increased volume of the former, thus making it far easier to establish its detailed anatomical features, although the terminology of both is essentially identical. The gland is said to be reddish-gray in color in the human and approximately ovoid in shape. It measures about 13 mm in its transverse diameter, about 9 mm in its antero-posterior diameter, and 6–9 mm in its vertical height. The pituitary in the human adult weighs about 0.4–0.9 g (average about 0.6 g), but this can double during

puberty, pregnancy, and even the menstrual cycle.[3] The anterior pituitary is said to account for about three quarters of its weight.[32] It is also evident that the meninges blend with the capsule of the gland and are not readily separated from it. For a diagram showing a coronal section through the pituitary gland, with an indication of its immediate relations in the human adult, see Thapar et al.,[33] and for another showing a sagittal section through the pituitary gland, indicating its relationship to the optic chiasm, see Miller and Newman.[34]

In the human, a "pharyngeal pituitary" may be present in association with a substantial palatal cleft and ocular hypotelorism. This is due to a complete or partial failure of ascent of RP from its site of origin. More commonly, however, only a small portion of the pharyngeal end of the stalk of RP may persist as a pharyngeal pituitary.[26] Alternatively, the lumen of RP may not be obliterated, which can result in the presence of cysts or cystic remnants at the interface between the anterior and posterior lobes. This is found in about 20% of pituitary glands at autopsy.[35,36] In the human, the remnant of the cavity of RP is invariably present at the time of birth but usually disappears during early childhood. Its site of ascent is sometimes present in the form of a craniopharyngeal canal. If present, the latter is located in the midline within the sphenoid bone and often opens into the base of the sella turcica. The pars intermedia is rudimentary in the human, and part, or occasionally most, of it may be displaced and even become incorporated into the pars nervosa. Observations on the typical histological features of the various parts of the gland are provided herein.

It is now well established from numerous experimental studies that at least seven distinct hormones are synthesized and released from the adenohypophysis, although only the roles of those not discussed elsewhere are briefly noted here. These include *somatotrophin* (STH), which is involved in the control of prepubertal body growth, and *mammotrophin* or *lactogenic hormone* (LTH), which is involved in the secretory activity of the female mammae during pregnancy. ACTH, TSH, FSH, *interstitial cell stimulating hormone* (ICSH), and MSH are discussed in applicable chapters in this volume.

Numerous recent studies have been undertaken in an attempt to establish the cellular sites of secretion of these and other hormones. Most of these studies have involved either immunohistochemical techniques or, more recently, molecular approaches in order to analyze the hypothalamic region and/or the pituitary glands isolated from individuals who had suffered from clinical conditions known to involve functional defects of either the hypothalamus or pituitary. A wide range of cell lineage studies were also undertaken to investigate hypothalamic and pituitary function, for example, by analyzing clinical conditions and tumors that produce an increase or decrease in the output of specific hormones.[3,6,7] An analysis of this material is beyond the scope of the present chapter.

THE BLOOD SUPPLY AND INNERVATION OF THE PITUITARY GLAND AND HYPOTHALAMUS IN THE HUMAN

The two internal carotid arteries ascend in the carotid canals in the petrous part of the temporal bone and then curve forward and medially where they enter the intracranial part of their course. Here, on either side of the sphenoid bone, the two internal carotid arteries provide the vascular supply to the hypothalamic region via a number of branches. A pair of *superior* hypophyseal arteries normally supplies the hypothalamus and forms a substantial capillary network within its median eminence and also the upper part of the pituitary stalk. The long hypophyseal portal vessels originate from the infundibular capillary plexuses, while the short hypophyseal portal vessels principally drain the region of the infundibular stalk. Between them, these vessels form the hypothalamic portal vessels and represent the main blood supply to the anterior pituitary gland, where they form a secondary plexus of sinusoidal capillaries that supply its secretory cells.

A contractile internal capillary plexus derived from stalk branches of the superior hypophyseal arteries regulates the release of hypothalamic hormones, and retrograde blood flow toward the median eminence also occurs. A pair of *inferior* hypophyseal arteries that arise from the internal carotid arteries when they are located within the cavernous sinuses forms the principal supply to

the posterior pituitary gland.[3] Efferent veins drain the anterior and posterior regions of the pituitary gland into the adjacent cavernous dural venous sinuses. Various studies indicate that there may be retrograde flow of hypothalamic and pituitary peptides via vessels along the pituitary stalk back to the brain. It has also been suggested that this may account for their presence in the cerebrospinal fluid.[32] A simplified view of the hypothalamus and its vascular connections between the anterior and posterior parts of the pituitary gland is shown in the chapter on the endocrine system in *Gray's Anatomy.*[1]

Much of the information on which the vascular supply of the pituitary gland, the median eminence, and the hypothalamus is based has been obtained from the analysis of preparations in which the vascular supply to this region has been determined following the injection of these vessels with latex. The corrosion casts were then examined by scanning electron microscopy. This approach has been taken by a number of researchers, and the information obtained from these sources is briefly alluded to in this section and in the section in this chapter, "The Pituitary Gland in the Adult Rat."

A considerable amount of information is now available about the innervation of the pituitary gland. As this undoubtedly plays a critical role in the functioning of the gland, in association with the complex vascular arrangements present, this is described in the section in this chapter, "The Control of Secretion of the Pituitary Hormones."

HISTOLOGICAL APPEARANCE OF THE VARIOUS PARTS OF THE PITUITARY GLAND IN THE HUMAN

For observations on the terminology used here, see the earlier section titled "Terminology." It is possible to distinguish among the various parts of the pituitary gland by their distinct histological appearance. The pars distalis component of the adenohypophysis, for example, is composed of large numbers of secretory cells that are either round or polygonal in shape. They are arranged in cords and "nests" and are separated by a rich fibrovascular sinusoidal network. Hematoxylin and eosin (H&E) stained sections allow its cellular components to be approximately equally divided into either chromophils or chromophobes. The chromophils are subdivided into either acidophils or basophils according to their staining properties. The pars intermedia region of the adenohypophysis is separated from the pars anterior by a cleft (the remnant of the lumen of RP) lined by cuboidal epithelial cells. The histological appearance of the pars intermedia allows it to be readily distinguished from the pars distalis because it consists of bands of pale staining polygonal cells that contain a foamy cytoplasm. These cells are also larger than the polygonal cells in the pars distalis.

The posterior lobe of the pituitary consists of the pars nervosa, which lies adjacent to the pars intermedia, and is a continuation of the infundibulum or pituitary stalk. The pars nervosa appears to be relatively eosinophilic and hypocellular and is composed of unmyelinated axons of neurosecretory neurons of hypothalamic origin. These terminate on numerous capillaries that are supported by modified glial cells or astrocytes (pituicytes).

THE PITUITARY GLAND IN THE ADULT RAT

The location and gross morphology of the pituitary gland and its relationship to the hypothalamus in the rat is generally similar to that in the human and in the mouse (see below), although there are clearly minor species differences. The difference between the sexes in the weight of the pituitary gland is particularly marked in the rat: the average weight is 8.9 mg in the male and 15.9 mg in the female.[37] This difference in this species is principally due to the greater weight of the anterior lobe in the female, where the volume of the three principal parts of the gland are said to be adenohypophysis (81.7%), pars intermedia (8.5%), and neurohypophysis (9.8%).[35] The dimensions of the gland vary among individuals and between sexes, but the width is normally about 4.5–5.5 mm, its length is about 3.0–3.5 mm, and its dorso-ventral height about 1.5 mm. It is also relevant to note that in the

rat the entire pituitary gland has a substantial blood supply, with wide sinusoids in the anterior lobe associated with numerous narrower capillaries in the posterior lobe.[38] A detailed description of the cytoarchitecture and connections between the hypothalamus and the pituitary in the adult rat is also available.[4] In the rat, the residual cleft, being the derivative of RP, remains throughout life.

The blood supply of this organ is considerable. Wide sinusoids consisting of dilated capillaries and numerous narrow capillaries are present in the posterior lobe, and in this species these are more numerous than those in the pars anterior.[38] The vasculature in the posterior lobe also forms a close network in contrast to the coarser network in the anterior lobe. The vessels in the posterior lobe are about half of the diameter of those in the anterior lobe and are consequently more difficult to see in uninjected preparations. Most of the pars intermedia is relatively avascular, containing only a few capillary loops, although its thickened periphery is relatively vascular. The latter vessels arise from the neural lobe. By contrast, the pars tuberalis has a rich blood supply. A number of studies have also explored the microcirculation within the gland (see below).[39-41] The blood supply and innervation of the pituitary has also been described in considerable detail.[39,42]

The nerve supply of the pars tuberalis is of two types.[4,43] Various staining studies have investigated the cellular morphology of the various components of the pituitary gland in the rat, and these are similar to those described previously for the human and the mouse.[4]

PRENATAL DEVELOPMENT OF THE PITUITARY GLAND IN THE MOUSE

Kaufman published one of the more descriptive accounts of the early stages in the development of the pituitary gland in the mouse,[14] and this was supplemented by additional studies on the sequential stages in the development of this organ.[44] More recently, the latter was expanded in the form of text-based anatomical databases that are continuously being updated (http://www.genex.hgu.mrc.ac.uk/, http://www.informatics.jax.org/). The account of the development of the pituitary gland presented here is largely based on the information provided in these various sources but was supplemented where indicated. As previously noted, most of the preliminary information relates to the development of this gland in the mouse, indicating the various components that are formed and are readily recognized in conventional histological sections stained with H&E at each standard developmental or Theiler stage (TS).[45,46]

HISTOLOGICAL LANDMARKS ASSOCIATED WITH THE APPEARANCE OF THE PITUITARY GLAND AT SEQUENTIAL STAGES OF DEVELOPMENT IN THE PRENATAL MOUSE[14]

TS 13–14: The buccopharyngeal membrane is first clearly recognized in embryos at about 8.5–9 dpc. However, even by this time this bilayered membrane is already in the process of breaking down. The latter is particularly clearly seen in median sagittal sections of embryos at this stage of development, and by about 9.5 dpc all traces of it have disappeared. The first indication of the ectodermal placode from which RP develops is also seen during the early part of this stage, in the rostral extremity of the oral cavity, in embryos with about 15 pairs of somites. The stage of development displayed here is very similar to the diagram shown in Schwind of a sagittal section through the cephalic region of a 19-somite rat embryo.[47]

TS 15: The first evidence of differentiation of the rostral or upper part of the wall of RP is seen in embryos with about 20–25 pairs of somites. It is also evident that the peripheral boundary of the entrance to the pouch, in the roof of the oropharynx, is much wider than observed at earlier stages of development. This increase in the circumference of the entrance into RP is particularly well seen when this region of the roof of the oropharynx is viewed by scanning electron microscopy. As RP ascends, any mesenchyme between

its rostral part and the floor of the diencephalon becomes progressively dispersed. Thus, when contact is eventually made between the future pars intermedia (of RP origin) and the floor of the diencephalon, no mesenchyme is seen to separate them in conventional histological sections, thus allowing the possibility of inductive interaction between them. No indication of differentiation of the floor of the diencephalon to form a placode-like structure is seen at this stage. The stage of development displayed here is very similar to the diagram shown in Schwind of a sagittal section through the cephalic region of a 27-somite rat embryo.[47]

TS 16–17: At about 10.25–10.5 dpc, in embryos with about 30–35 pairs of somites the first evidence of an infundibular recess is seen. While no evidence of a lumen is observed within the primordium of the future posterior pituitary at this stage, the walls appear to be indistinguishable from the neural ectoderm in the region of the floor of the diencephalon at this site. While the lateral walls of RP appear to be uniform in thickness, its most distal part, in the region of the future pars intermedia where it makes contact with the wall of the infundibular recess, is somewhat thinner than elsewhere. The stage of development displayed here is very similar to the diagram shown in Schwind of a sagittal section through the pituitary gland of a 12-day, 6-hour rat embryo (approximately 27-somite stage).[47]

TS 17: In embryos with about 35–40 pairs of somites, a substantial narrowing of the entrance to RP is clearly seen at this stage when compared to the situation observed at about TS 15, particularly when this region is viewed by scanning electron microscopy.

TS 18: At about 11–11.5 dpc, in embryos with about 40–45 pairs of somites the infundibular recess is now seen to be both wide and deeply indented at its entrance in the floor of the third ventricle, and its lumen extends distally toward the tip of the recess. Also apparent at this stage is the more constricted than previously width of the entrance to RP from the oropharynx[1]; in contrast, its lateral walls appear to be uniformly differentiated, and the diameter of its lumen seems to be uniform throughout its length. Only at its distal tip, where RP makes contact with the tip of the infundibular recess, can an indentation now clearly be seen. It is now no longer possible to recognize the site of the former buccopharyngeal membrane, as the ectoderm of the oral cavity appears to be in complete continuity with the most rostral part of the pharyngeal endoderm.

TS 19: At about 11.5–12 dpc, in embryos with about 43–48 pairs of somites the entrance to the infundibular recess appears to be slightly wider from side to side than formerly. In the case of RP, while its entrance site from the oropharynx has almost completely disappeared, the side-to-side width of the pouch is considerably greater than formerly, and the impression is gained that part of its anterior wall is slightly thicker than its posterior wall. Its lumen is also particularly obvious at this stage.

In advanced TS 20 stage embryos: At about 12.5 dpc, in embryos with about 50 pairs of somites it is now evident that certain changes are occurring in the morphology of the pituitary gland. This is particularly well seen when a median sagittal section is viewed. For example, a remnant of the stalk that formerly connected the roof of the oropharynx with the ventral part of RP is clearly seen. The stage of development displayed here is very similar to the diagram shown in Schwind of a sagittal section through the pituitary gland of a 14-day, 12-hour rat embryo.[47] Around this area, some evidence of mesenchyme condensation is seen that will soon form the precartilage mass of the sphenoid. In the region of the pars intermedia, the thickness of the wall of RP remains unchanged compared with the situation observed formerly. The width of the lumen of RP also remains fairly uniform throughout the transverse diameter of this part of the gland. The entrance to the infundibuilar recess gradually narrows compared with the situation observed formerly. Obvious evidence of pars tuberalis formation is also observed at this stage, with the growth of the supero-lateral "wings" of the pars anterior on either side of the infundibular stalk.

TS 22: At about 13.5 dpc, in embryos with about 60 pairs of somites there is some evidence of proliferative activity when sagittal sections through the pars anterior region of the pituitary gland are observed at this stage of its development. Similarly, between the gland and the roof of the oropharynx, early evidence of precartilage condensation is seen. In intermittent transverse sections through this region, considerable evidence of proliferative activity is now seen in the ventral part of the pars anterior. Increasing growth of the pars tuberalis around the infundibular stalk is also apparent at this stage. A substantial lumen is still present in the region of the pars posterior at this stage. The two internal carotid arteries are located one on either side of the sphenoid bone, and these provide the vascular supply to the hypothalamic region via a number of its branches. These vessels form the hypothalamic portal vessels and the main blood supply to the anterior pituitary gland. It has now been established that a pair of *superior* hypophyseal arteries supplies the hypothalamus. These vessels form a substantial capillary network within its median eminence and also supply the upper part of the pituitary stalk. The long hypophyseal portal vessels originate from the plexus within the median eminence, while the short hypophyseal portal vessels chiefly drain the region of the infundibular stalk. Between them, these vessels form the hypothalamic portal vessels and the main blood supply to the anterior pituitary gland. Here they form a secondary plexus of sinusoidal capillaries that supplies its secretory cells.

TS 22–23: At about 14.5 dpc, the most obvious difference compared to the previous stage of development is the dramatic increase in the amount of cellular and vascular proliferative activity observed in the anterior wall of RP. This is the region that gives rise to the pars anterior. By contrast, there is no evidence of similar activity in the posterior wall of RP, the part that forms the pars intermedia. What is also evident at this stage is an increase in the degree of proliferative activity in the wall of the pars nervosa, although a considerable lumen is still present in this region of the pituitary. Differentiation is now evident in the precartilage condensation of the sphenoid, and this is largely replaced by the cartilage primordium of this part of the base of the skull.

TS 24: At about 15.5 dpc, further evidence of cellular and vascular proliferative activity is observed in the pars anterior, and the volume of this part of the pituitary has increased substantially compared to the previous stage. The increased cellular activity in the region of the pars nervosa also results in the progressive obliteration of its lumen, although the entrance to the infundibular recess is still clearly seen. While the basisphenoid is now completely cartilaginous, early evidence of ossification is seen in the region of the floor of the sella turcica, and a considerably larger area of ossification has developed more caudally within the parachordal plate.[14] The cartilage primordia of the pre- and post-sphenoid bone are clearly seen in sagittal sections of this stage of development and are located at a different, and slightly more rostral, site from the craniopharyngeal canal seen in sagittal sections of the pituitary gland shown at TS 25. It is also very similar to the diagram shown of a sagittal section through the pituitary gland of a 15-day, 24-hour rat embryo displayed by Schwind.[47]

TS 25: At about 16.5 dpc, further evidence of cellular and vascular proliferative activity is observed in the pars posterior, and this appears to have resulted in the obliteration of its lumen, although the entrance to the infundibular recess is still clearly recognized. It is also possible to identify in this sagittal section what appears to be a small "rest" of tissue that probably represents a "pharyngeal pituitary." This is located at the site where the neck of RP has failed to separate from the roof of the oropharynx. This also appears to be associated, rostrally, with a craniopharyngeal canal.[48] It is characteristically located slightly more caudally than the "sphenoidal" canal[28] observed previously.

TS 26: At about 17.5 dpc, the lumen of the pars nervosa has now completely disappeared, due to the increased level of cellular and vascular proliferative activity observed at this site, and the infundibular recess is also less marked than previously. The stage of development

displayed here is very similar to the diagram shown in Schwind of a sagittal section through the pituitary gland of a 19-day, 12-hour rat embryo.[47] The residual lumen of RP is clearly seen at this stage, as is the meshwork of cells surrounding the vascular plexus that develops within the pars anterior of the pituitary gland. The pars tuberalis almost completely surrounds the infundibular stalk at this stage.

GROSS ANATOMY OF THE PITUITARY GLAND IN THE ADULT MOUSE

An account of the gross anatomy of this gland in the adult mouse provides a useful backdrop to an analysis of its developmental features, because this necessarily also draws attention to the terminology used to describe its various parts.[49] The pituitary gland (the hypophysis cerebri) is located in the midline in an indentation in the dorsal surface of the sphenoid (or basisphenoid) bone. This is one of the principal components of the base of the skull, formed from the cartilaginous basicranium. The pituitary gland in the mouse is situated in a small indentation of the sphenoidal bone termed the sella turcica (or hypophyseal fossa) because its shape in the human is said to be similar to a Turkish saddle. In the adult human, but not in the mouse, the sella forms the thin bony roof of the sphenoid sinus.

While the terminology used in the various texts written on the *neural* part of the pituitary (or the neurohypophysis) vary widely, *Gray's Anatomy* favors the use of the terms *neurohypophysis* and *adenohypophysis*, as both include parts of the infundibulum and the former terms *anterior* and *posterior* lobes do not. According to this source, the term neurohypophysis includes the median eminence, infundibular stem and neural lobe or pars posterior. Surrounding the infundibular stem is the pars tuberalis, a component of the adenohypophysis. These two terms are therefore defined as follows. The neurohypophysis includes the pars posterior (pars nervosa, posterior or neural lobe), infundibular stem, and median eminence. The adenohypophysis includes the pars anterior (pars distalis or glandularis), pars intermedia, and pars tuberalis.[1] The neurohypophysis is connected to the hypothalamic region at the base of the brain by a thin stalk (the infundibulum, infundibular stem, or pituitary stalk). On an inspection of the ventral surface of the brain, it is evident that this part of the pituitary gland is connected to the median eminence of the *tuber cinereum* just caudal to the optic chiasm. Most authorities now consider that the neurohypophysis includes the median eminence, the infundibular stem, as well as the posterior part of the gland (the neural lobe, pars nervosa, or pars posterior). While the pars nervosa initially has a central lumen, this is soon lost.

The gland is oval in shape with its long axis perpendicular, i.e. at right angles, to the long axis of the skull. On superficial inspection, the gland is divided into a number of principal parts. The centrally and posteriorly directed pars nervosa in this species is almost completely surrounded by the pars intermedia, and the most laterally and ventrally located part of the gland is termed the pars distalis (the anterior or distal lobe). A relatively small part of the anterior lobe (the pars tuberalis) extends on either side of the pituitary stalk, being partially wrapped around it. As stated previously, the term adenohypophysis is commonly used to include the pars distalis, pars intermedia, and pars tuberalis.

The pars nervosa is more opaque in appearance than the other regions of the gland in humans but not in mice, and is derived from the region of the infundibular recess of the diencephalon, in the floor of the third ventricle of the forebrain. The other two regions of the gland are derived from an evagination of the roof of the oral cavity, termed RP. As the following description of the development of the pituitary gland describes, both its neural and oral components are ectodermally derived. While under normal circumstances the connection between RP and the roof of the oral cavity is soon lost, remnants (usually nonmalignant) may be located in the adult along its path of ascent; occasionally, these "rests" of cells may present as malignant tumors. In some cases, a "pharyngeal pituitary" may be present in the median plane in the roof of the oral cavity, and may even extend the full length of the craniopharyngeal canal.

The pituitary gland in the female mouse is consistently larger and heavier than in males of the same age and strain. This may be by as much as 0.5–1.5 mg,[50] where the normal wet weight in the

adult is about 1–3 mg and its dry weight is usually about 0.30–0.85 mg. There are also considerable strain differences, however, in the weight of this gland.[50]

While not readily seen in the mouse, both in the rat and in the human the pituitary fossa is covered by a thin membrane consisting of dura mater (the diaphragma sellae) through the central part of which the infundibulum passes to the posterior pituitary. The infundibulum is the only part of the gland covered by pia mater, as elsewhere the dura is fused to the capsule of the pituitary gland.[51]

HISTOLOGICAL FEATURES OF THE VARIOUS PARTS OF THE PITUITARY GLAND IN THE MOUSE

The largest portions of the pituitary gland in the mouse are those derived from RP: pars distalis, pars intermedia, and pars tuberalis. Each region of the gland has a quite distinctive cellular morphology. It has been noted that the size of the granules in the cells within the pituitary varies considerably, and this has allowed the various cell types to be distinguished at the ultrastructural level. While the somatotrophs and prolactin-secreting cells tend to have the largest granules, the thyrotrophs tend to have the smallest granules. This is not, however, a particularly reliable method, as the volume of these granules tends to vary somewhat according to the secretory status of the cell.

Pars Distalis

The pars distalis has a bilobed structure most easily seen on transverse sections through the gland and contains polygonal secretory cells arranged in cords and nests; an extensive fibrovascular network separates these. The cell types recognized in this region of the gland are *chromophils* (acidophilic and basophilic according to their staining characteristics) and *chromophobes*, each constituting about half of the cell population present in this part of the gland. The cytoplasm of the chromophilic cells characteristically contains secretory granules. These granules are not present in the chromophobic cells. Within the pars distalis are the (nonsecretory) *folliculo-stellate cells*, and these form a relatively small proportion of the cells found in this location. These are morphologically similar to dendritic cells and display variable immunoreactivity to the S100 protein (a dendritic cell marker). It is within the pars distalis that the majority of the anterior pituitary hormones are produced and from which they are secreted, and in this regard the situation in the mouse is similar to that described in other mammalian species.

It has been suggested that somatotrophs and mammotrophs are derived from the same cell lineage, and mammosomatotrophs (that secrete both GH and PRL) have been demonstrated in both the mouse and in other mammalian species.[52] The basophilic gonadotrophs (that secrete both FSH and LH) are more numerous in males than in females, while in females these cells are more frequently seen during proestrus.

Pars Intermedia

This part of the anterior pituitary gland is separated from the pars distalis by the cleft representing the remnant of RP. Similar to the rat pituitary intermediate lobe,[53-55] proliferation of melanotrophs decreases after the first postnatal day (P1) 1 and ceases almost completely between P 35 and 60.[56] Within the pars intermedia, the cells are polygonal in shape, pale staining, and contain "foamy" cytoplasm. These cells are arranged in nests and are larger than the polygonal cells present in the pars distalis.[5] All these features allow this region of the gland to be readily distinguished histologically from the pars distalis, when the two appear to be closely adherent. It was also noted that the proportional volume of the pars intermedia in the mouse is greater than that in most other species. This region of the gland is the principal source of ACTH, MSH, and β-endorphin.[57] While the corticotrophs (which secrete ACTH) constitute the largest component of cells in this region of the gland, such cells are also scattered throughout the pars distalis.

Pars Nervosa (Neurohypophysis)

This region of the gland, as noted previously, consists principally of the posterior lobe (pars posterior) and the infundibular stalk. The cells of the pars nervosa often appear to be adherent to those of the pars intermedia and pars tuberalis at their site of contact. The constituent cells of the pars nervosa are relatively eosinophilic, and this region of the gland also appears to be hypocellular. Many of its cells are neurosecretory neurons of hypothalamic origin. The latter cells terminate on the large number of capillaries supported by the modified glial cells (pituicytes) present in this region of the gland.

THE CONTROL OF SECRETION OF THE PITUITARY HORMONES

As the control of secretion of the various pituitary hormones is so complex, it is possible to provide only a brief overview here of the general principles involved. There are clearly two principal factors involved: (1) the innervation to this region from the hypothalamus; and (2) the complex vascular arrangements present, termed the hypothalamo-hypophyseal portal system. As the anterior pituitary lacks its own nerve supply, the neural control is regulated by factors (termed releasing hormones) secreted by neurosecretory neurons of the hypothalamus. The releasing hormones are transported to the pituitary where they are released into the upper part of the capillary plexus of the venous portal system. They are then carried to the adenohyphysis, where they in turn act on its endocrine producing cells. Releasing hormones have been recognized for each of the various hormone-producing cells of the adenohypophysis. The amount of any particular hormone, such as TSH, FSH, LH, or ACTH, released by the appropriate trophic cells within the anterior, such as pituitarythyrotrophs, gonadotrophs, or corticotrophs, is controlled by the blood concentration of the hormones produced by the relevant target organs. These release appropriate substances into the blood that then pass back to the hypothalamus. Thus, for example, the TSH secretion produced by the thyrotrophs of the anterior pituitary is controlled (usually inhibited) by the amount of triiodothyronine (T3) or thyroxine (T4) produced by the target organ (in this case, the thyroid gland) by means of a complex feedback loop.

The actual mechanism of release of the individual hormones into the circulation is via the exocytosis of the contents of vesicles from appropriate trophic cells that are located largely within the anterior pituitary. These hormones are released into local sinusoids and thus into the bloodstream. Whether they are released as well as the amount released is under the control of the releasing factors from neurons located in the median eminence, the arcuate nucleus, and other hypothalamic nuclei. The releasing factors pass into the upper part of the capillary bed of the venous portal system from where they are carried to the appropriate endocrine-producing cells. The neural signals are then converted or transduced from one type of signal to another. The neurons that produce the initial signals are peptidergic, whereas those that modulate the release of hormones are largely monoaminergic. Proliferation and maturation of melanotrophs are negatively controlled by hypothalamic dopaminergic innervation projected from the neural lobe during first days of the postnatal development.[54,55,58,59] Those wishing to obtain a more detailed analysis of the factors that control the secretion of individual hormones by the pituitary are recommended to read some of the numerous accounts on this topic.[1,3,5,7]

THE MOLECULAR FACTORS BELIEVED TO PLAY A ROLE IN THE ESTABLISHMENT OF THE HYPOTHALAMO–PITUITARY AXIS

Relatively little information appears to have been published on the early features of the pituitary gland in the mouse that provides detailed information on what may be observed at specific Theiler stages of its development.[14] General observations on the histological development of this gland in a number of other mammalian species such as in the human[1,10,16] and albino rat[47] are, however,

available, although an enormous amount of information was recently published on the molecular events that occur during its early development in the mouse.[6] By using the latter approach and subsequent studies, it was possible to establish the earliest factors believed to control both the anterior-posterior and dorsal-ventral axes of the embryo. The earliest information relevant to the development of the pituitary gland was obtained from fate mapping studies involving the analysis of experimentally produced chick-quail chimeras. It was suggested that information obtained from these studies may shed important light on the initial development of the pituitary gland. These studies have demonstrated, for example, that during embryogenesis the *anterior neural ridge* (or plate) of the early embryo gives rise to the anterior pituitary, the nasal cavity, and the olfactory placode. Similarly, these studies indicated that the most *posterior part of the neural plate* gives rise to the hypothalamus, posterior pituitary, optic vesicles, and ventral part of the forebrain.[61–63]

More recent studies confirmed what has long been suspected: Inductive interactions between the oral ectoderm and the overlying neural ectoderm also play a role in the subsequent development of the pituitary.[64] At a later stage in the development of the pituitary, a variety of stimulatory and inhibitory factors produced by the hypothalamus are believed to be involved in regulating the proliferation of each of the various cell types present in the hypothalamo-pituitary axis, according to the needs of the organism.[6]

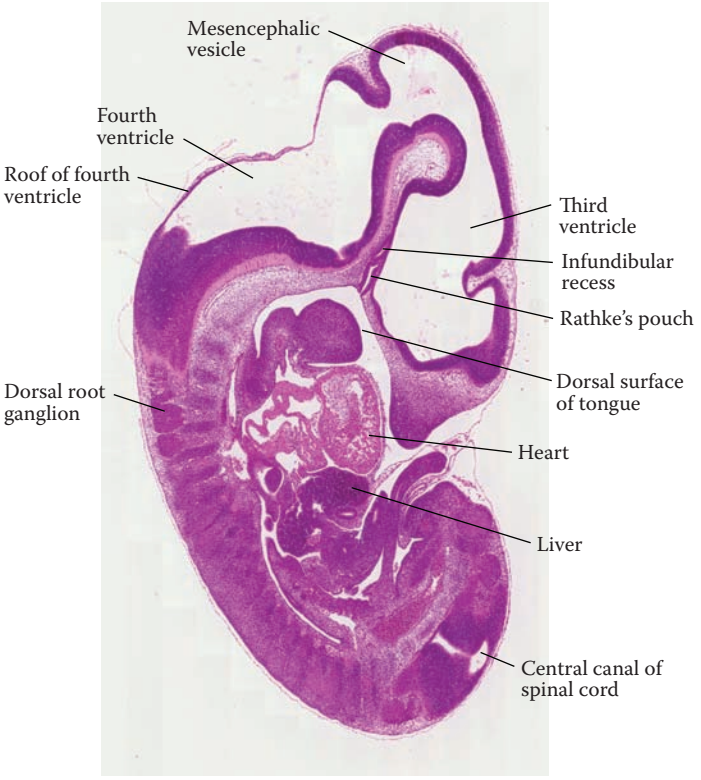

FIGURE 3.1 (E11_Fig3.1.svs) Low magnification, sagittal section through the right side of a mouse embryo (E11–11.25) stained with hematoxylin and eosin (H&E). Note in particular that the roof of the oropharynx is sectioned close to the median plane and clearly displays the third ventricle, the mesencephalic vesicle, and the rostral part of the fourth ventricle of the brain. The RP and infundibular recess of the third ventricle of the brain are clearly seen.

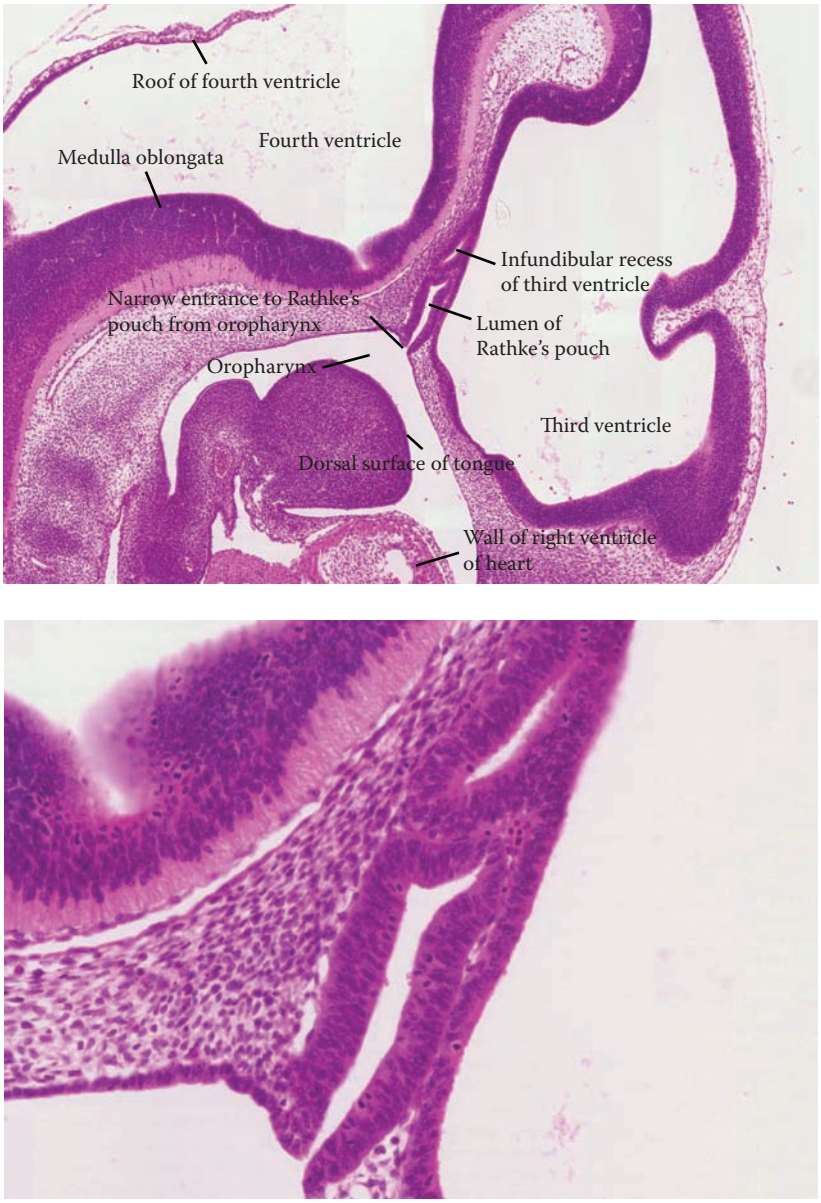

FIGURE 3.2 Medium (top) and high (bottom) magnification of Figure 3.1 illustrates the infundibular recess of the third ventricle of the brain and its close association with the rostral part of the wall of RP. Note that there is still a narrow connection between the caudal part of the lumen of RP and the roof of the oropharynx.

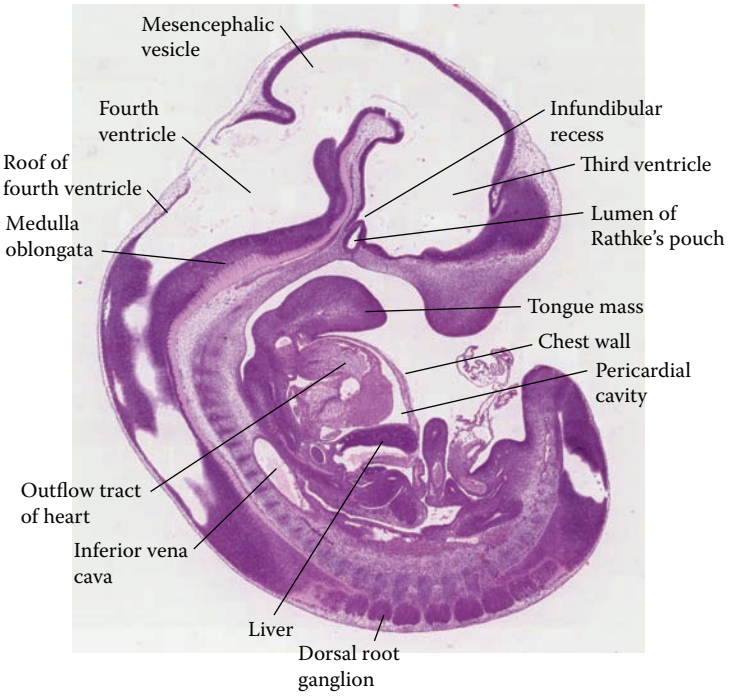

FIGURE 3.3 (E11.5_Fig3.3.svs) Low magnification, median sagittal section through the roof of the oropharynx and the base of the third ventricle of the brain of a mouse embryo (E11.5) stained with H&E. The lumen of RP is seen to have lost its connection with the roof of the oropharynx. The caudal extent of the fourth ventricle of the brain is now more clearly seen than in Figure 3.1.

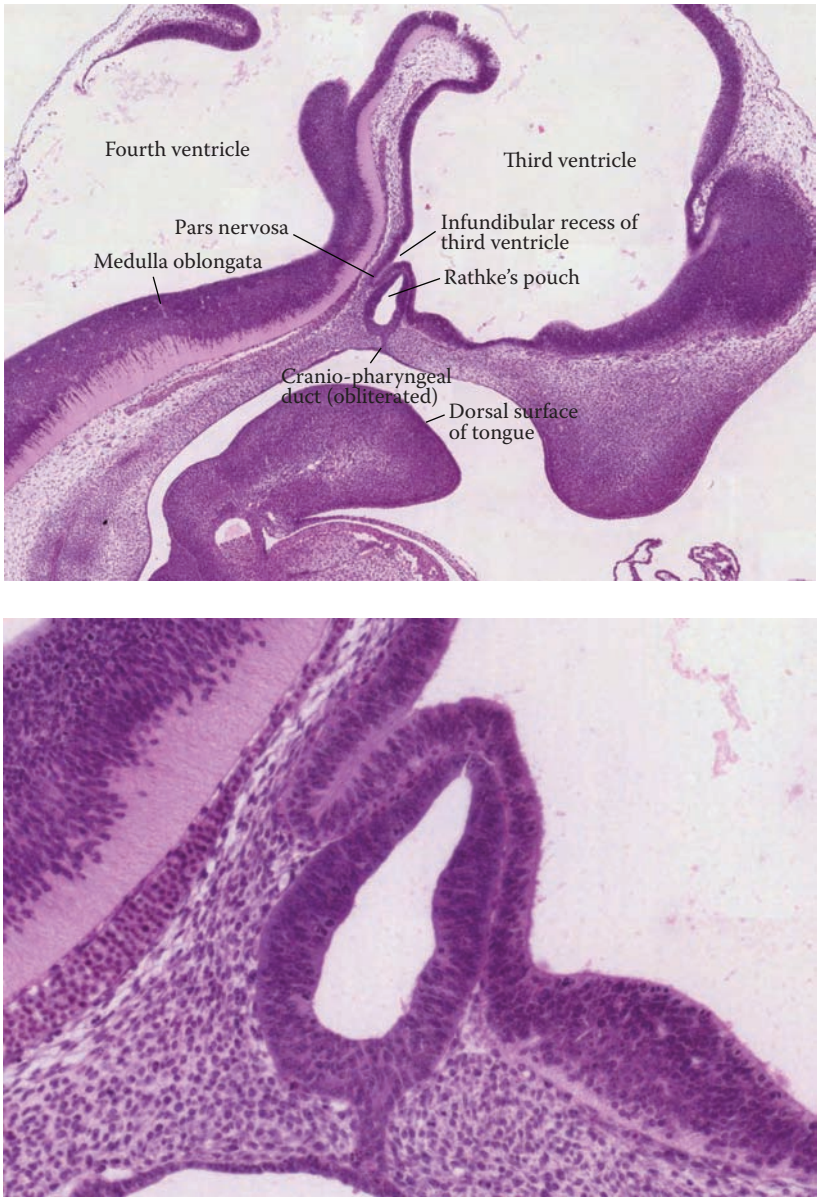

FIGURE 3.4 Medium (top) and high (bottom) magnification of Figure 3.3 illustrates the early development of the pituitary gland. Its two principal components that form the lumen of much of the anterior pituitary, RP, has now lost its connection with the roof of the oropharynx, and this obliterated region is represented by the cranio-pharyngeal duct, while the entrance from the floor of the third ventricle (its infundibular recess) is now clearly seen. This region will form the posterior part of the pituitary gland, while its wall forms the pars nervosa. The dorsal surface of the tongue is also clearly seen in the medium magnification image.

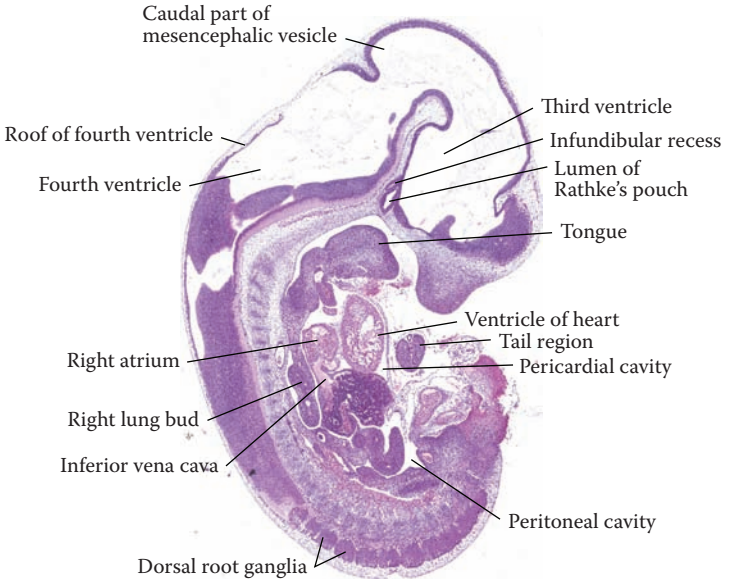

FIGURE 3.5 (E11.5_Fig3.5.svs) Low magnification, sagittal section close to the median plane through the roof of the oropharynx and the base of the third ventricle of the brain of a mouse embryo (E11.5–12) stained with H&E. The lumen of RP and the infundibular recess of the third ventricle are clearly seen.

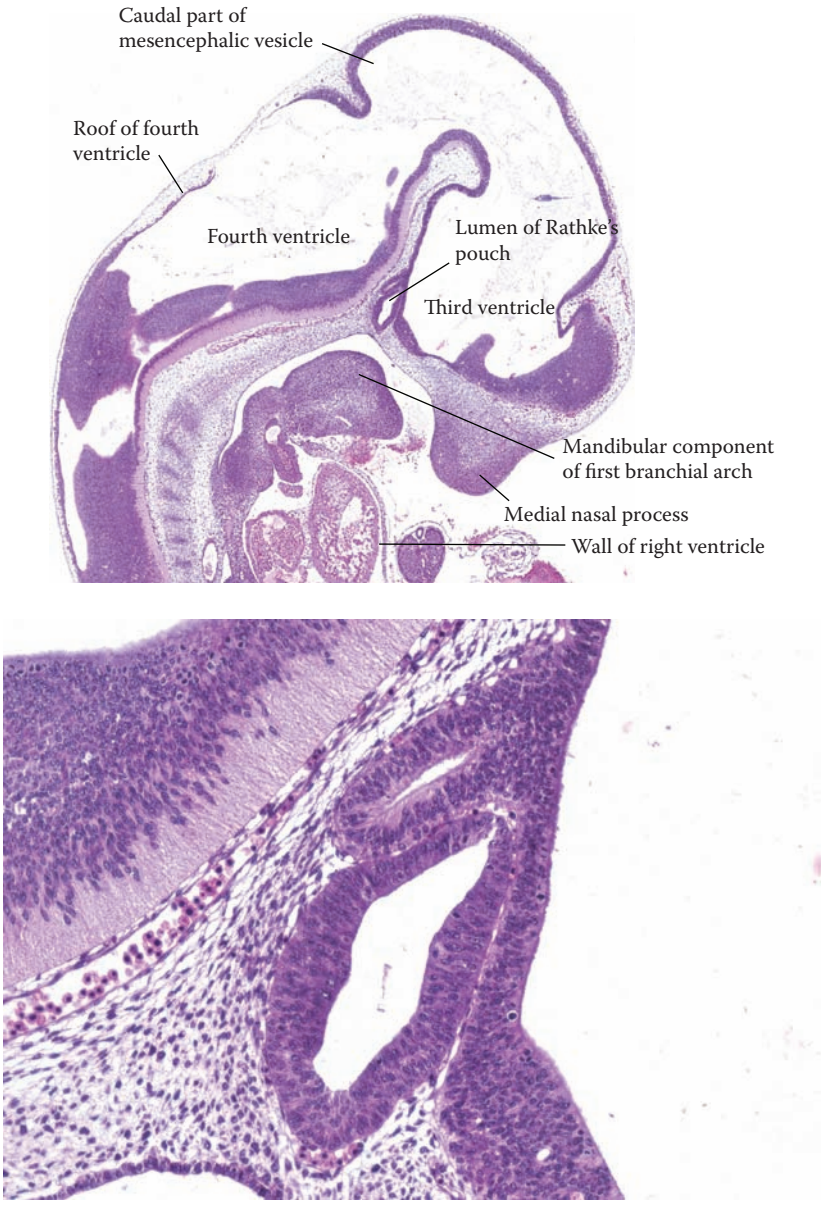

FIGURE 3.6 Medium (top) and high (bottom) magnification of Figure 3.5 shows the region of the future pituitary gland. Note in particular that the cranio-pharyngeal duct is no longer seen and that the infundibular recess between the third ventricle and the posterior pituitary appears to be obliterated.

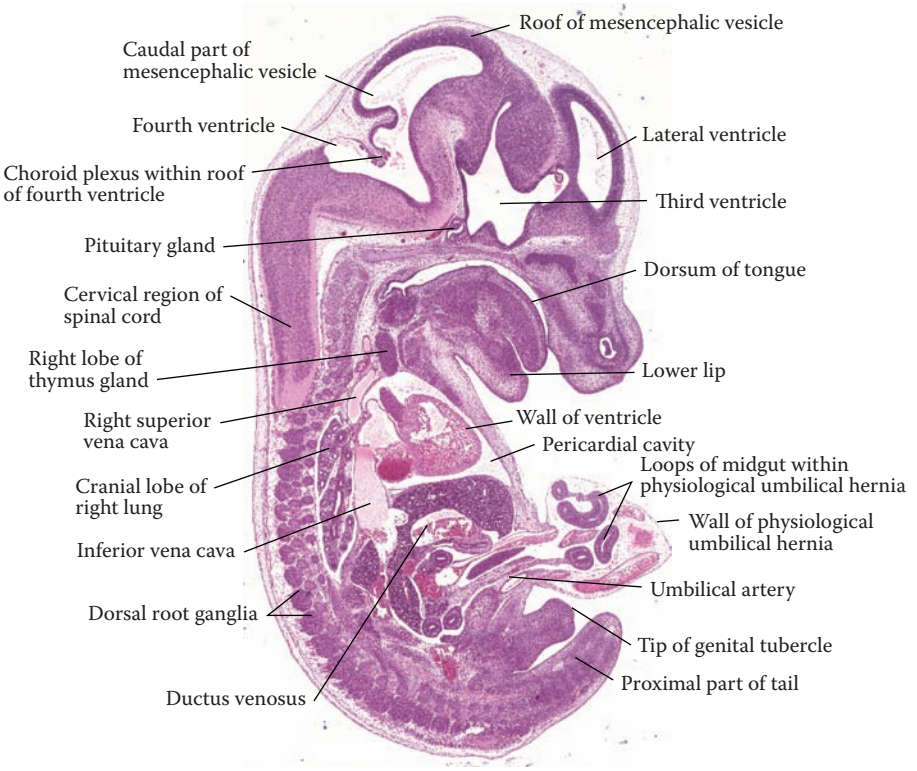

FIGURE 3.7 (E13.5_Fig3.7.svs) Low magnification, close to the median sagittal section through the roof of the oropharynx and base of the brain of a mouse embryo (E13.5–14) stained with H&E.

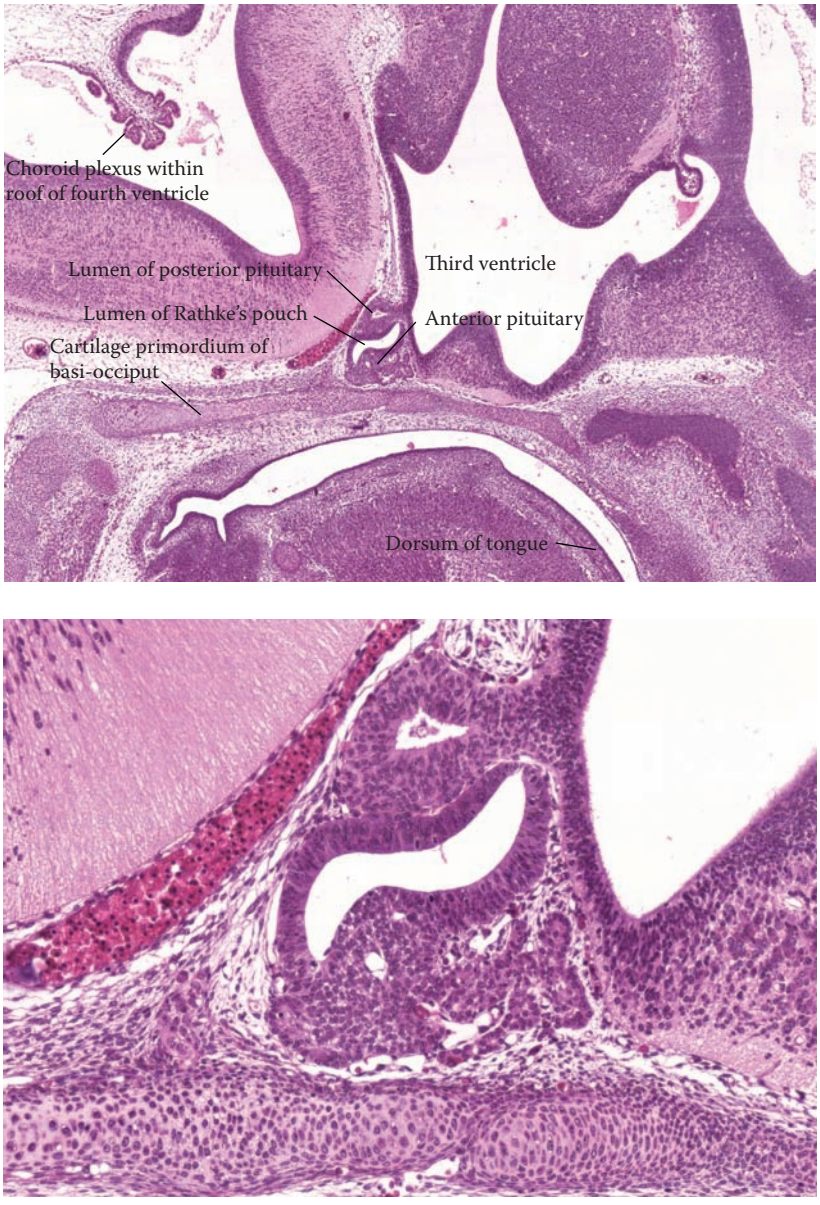

FIGURE 3.8 Medium (top) and high (bottom) magnification of Figure 3.7 illustrates the region of the pituitary gland at this stage of development. Evidence of differentiation of the anterior pituitary is clearly seen just caudal to the lumen of RP, while the lumen of the future posterior pituitary is also clearly seen, as is the cartilage primoridium of the basi-occiput.

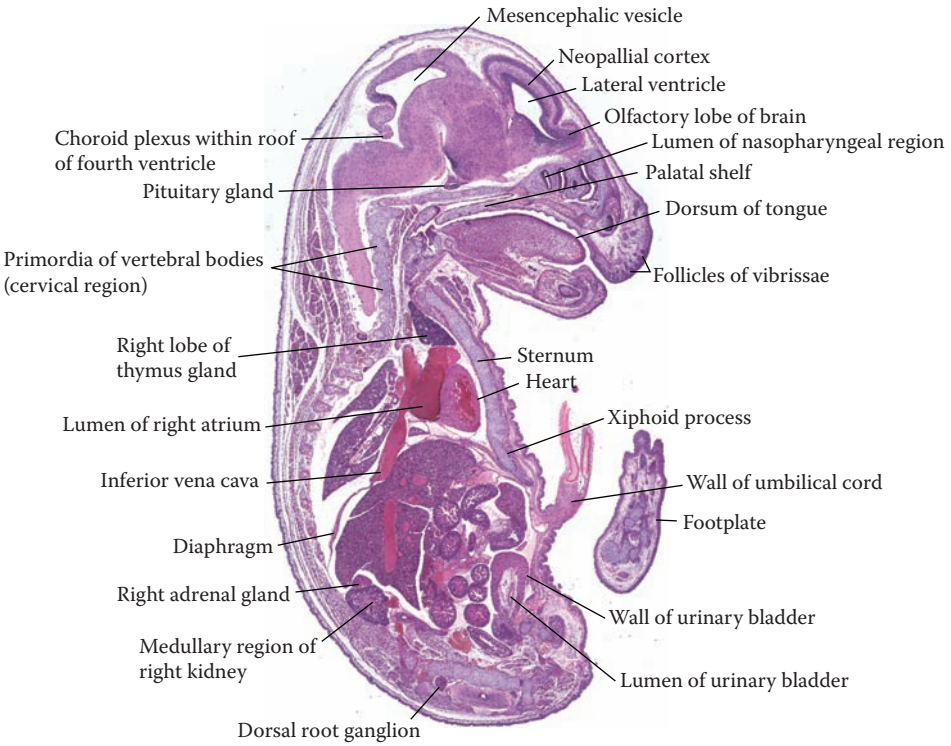

FIGURE 3.9 (E17.5_Fig3.9.svs) Low magnification, sagittal section through the nasopharyngeal and oropharyngeal regions of a mouse embryo (E17.5–18) that displays the base of the brain and pituitary region. This section is stained with H&E.

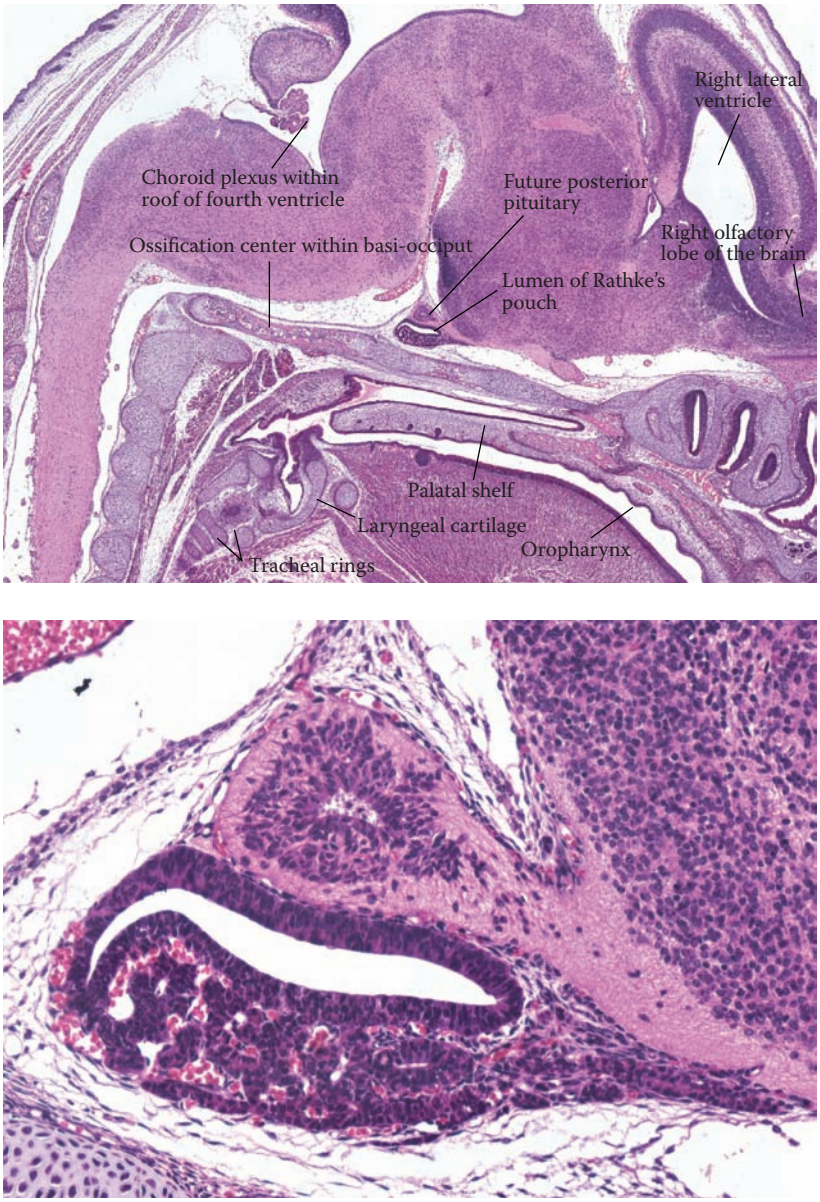

FIGURE 3.10 Medium (top) and high (bottom) magnification of Figure 3.9 showing the region of the pituitary gland at this stage of development. The lumen of RP has diminished in volume compared with the situation displayed in Figure 3.8, while the region of the future posterior pituitary gland is also clearly seen and the lumen of the future posterior pituitary gland appears to have diminished in size.

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4 The Thyroid Gland

INTRODUCTION

An enormous number of books and a far greater number of articles have been published on different aspects of thyroid gland development, anatomy, histology and ultrastructural morphology, physiology, and clinical aspects of thyroid disease. The principal problem encountered herein therefore has been to determine what material should be selected for insertion and what could be omitted. Accordingly, care has been taken to retain only material that is likely to be relevant to those whose interest is almost exclusively likely to be in the development and anatomical features of the mouse thyroid gland. This is principally because the mouse is now used almost exclusively as the model of choice by molecular biologists and geneticists who wish to investigate the factors that influence mammalian thyroid development and function. As in the consideration of some of the other endocrine glands described in this volume, most of the early studies have been undertaken in the human, and only relatively recently have comparable studies been undertaken in other mammalian species. These include rodents, and only very recently have studies been undertaken using the mouse as the mammalian model of choice. This is because of the very rapid expansion in molecular methodology to which this species is ideally suited.

It is for this reason that an overview is presented here of some of the more critical information that has been accumulated from the analysis of human embryos, fetuses, and postnatal material. Complementary information gained from the analysis of other mammalian species commonly studied by experimental embryologists and anatomists is then considered. It is also appropriate to consider here some of the most recent information that has been obtained on the molecular factors believed to play an important role in the development and physiological activities of this gland. Only where considered of critical importance in facilitating the understanding of the function of this gland is information provided on the common range of diseases and tumors involving the thyroid gland—that is, when such information sheds light on its abnormal morphogenesis and functional capacity. Because of its substantial size, the thyroid gland has been studied over many years, and more information has been written about it than most of the other endocrine organs considered in this book. Accordingly, the principal difficulty encountered herein has been in restricting the material covered to areas that are strictly relevant to the audience likely to wish or need to read its contents.

Much information is known about the early organogenesis of the human thyroid gland, and this and its histological and ultrastructural features are relatively easily covered here. Clearly, and for obvious reasons, less information is available on comparative aspects of the early development in the mouse, although much has been written about its development and features in, for example, the submammalian vertebrates.[1] Because of the relatively recent explosion in the number of molecular studies undertaken, it has to be appreciated that while some aspects of current knowledge on the subject are reviewed here, this represents only a very small proportion of the available information on this topic. Much of this is likely to be superseded over the next few years and even probably demonstrated to be incorrect. This is unfortunately inevitable in any very rapidly moving field where the methodology is advancing at an extremely rapid pace. While the descriptive material presented here is likely to stand the test of time, it is inevitable that this may not be the case with regard to the findings obtained from the leading edge of the field.

In the section titled "The Physiological Functions of the Thyroid Gland," attention will be drawn to the fact that the thyroid gland secretes three hormones. *Thyroxine* (T4) and *triiodothyronine*

(T3) regulate the metabolism of the body, while *calcitonin*, released by the parafollicular or C-cells of ultimobranchial body origin, controls the concentration of calcium in the extracellular fluid of the body. This endocrine gland is unique in that the storage of T4 and T3 occurs in the lumen of numerous spherical, cyst-like follicles that are bounded by a monolayer of simple cuboidal epithelium (in quiescent or synthesizing follicles) or columnar epithelium (in actively secreting follicles). These two hormones are stored in the semifluid *colloid* found within these follicles. It is present in the form of a high molecular weight glycoprotein called *thyroglobulin*. The latter is hydrolyzed to release these two hormones when it is removed from the lumen of the follicle by the process of endocytosis. This is followed by the intracellular action of lysosomal proteases. The source of these two hormones is also briefly considered here.

EVOLUTION OF THE MAMMALIAN THYROID GLAND

The thyroid gland appears to have evolved as an outgrowth from the rostral and ventral part of the primitive pharyngeal region of the foregut. Its principal function is to concentrate iodine and to synthesize and secrete hormones such as thyroxine, being under the control of hormones from the hypothalamic-pituitary axis. It has been suggested that the vertebrate thyroid probably evolved from a salivary-gland-like organ into an endocrine gland. The specific functions of the thyroid gland vary between species, but in all species studied it appears to play a critical role in the growth and development of the individual, especially of its nervous system. It also continues to play an important role in controlling growth throughout the preadult period and is most active in association with periods of rapid growth and development. With the amalgamation of the parafollicular or C-cells of neural crest and ultimobranchial body origin and their dissemination throughout the thyroid, this gland is then able to play an additional and equally critical role in maintaining calcium homeostasis in the body via the controlled release of thyrocalcitonin. For brief observations on the part played by the secretion of parathyroid hormone (PTH) and thyrocalcitonin (or calcitonin) released by the thyroid gland, in maintaining calcium homeostasis, see Chapter 5 in this volume.

DEVELOPMENT OF THE THYROID GLAND IN THE HUMAN EMBRYO

O'Rahilly has provided one of the most comprehensive, albeit brief, overviews of the sequence of development of the various endocrine organs during the embryonic period proper.[2] This covers the period between conception and about 8 weeks of gestation (termed Carnegie Stages [CS] 1–23). While opinions differ slightly in the literature, the first evidence of a thyroid primordium is seen in early somite-containing human embryos at about 20–22 days *post coitum* (dpc) (about CS 9–10). Most embryologists are of the opinion that the first evidence of this structure is probably seen in embryos with about seven pairs of somites.[3,4] Initially it appears as a specialized "plaque" or endodermal thickening in the midline in the floor of the pharynx, just caudal to the tuberculum impar. This thickening soon proliferates and forms into a diverticulum, and this is then referred to as the primordium of the thyroid gland.[5,6] At this very early stage in its development, the latter is in direct contact with the wall of the subjacent aortic sac and is located between the diverging first aortic arch arteries. It takes origin from a small indentation termed the *foramen cecum* situated in the midline between the first and second pharyngeal arches. The site of the foramen cecum is later seen as a relatively small midline indentation located between the posterior margin of the anterior two thirds of the tongue and the anterior part of the posterior one third of the tongue. The anterior one third of the tongue is formed principally from the first pharyngeal arch material with a relatively small contribution from the second arch, while the majority of the posterior two thirds is principally formed from mesoderm of third pharyngeal arch origin. The latter part of the tongue also contains a caudal contribution from the fourth arch. The anterior and posterior parts of the dorsum of the tongue are separated by the terminal sulcus. When the embryo possesses about 14 pairs of somites,

the caudal part of the diverticulum initially becomes flask or pouch shaped and its neck, where it is in continuity with the buccal cavity, and remains relatively narrow.[7]

When the embryo possesses about 17 pairs of somites, the principal part of the thyroid primordium grows caudally, and its connection with the floor of the pharynx is represented by a narrow hollow stalk-like connection termed the *thyroglossal duct*. The elongation of the thyroglossal duct and caudal migration and growth of the thyroid diverticulum as well as the associated development of the neck that occurs at this stage are most often due to the "descent" of the heart.[8] The thyroglossal duct soon becomes a solid tubular cord of tissue, while the most caudal and terminal part of the diverticulum destined to form the gland becomes bilobed. The duct then descends in the tissues in the front of the neck,[9] and shortly afterward, at about 32–33 dpc (at about CS 14–15), it usually loses its connection with the floor of the pharynx. It is also evident that the now solid thyroglossal duct tends to fragment. Shortly afterward, once the thyroid has lost its continuity with the pharynx it is clearly seen to possess two lobes that are united across the ventral midline by an isthmus and, at this stage, frequently also has a short median remnant. If the latter is retained, it probably forms the "pyramidal lobe" of the gland. After the thyroglossal duct loses its connection with the pharynx, the proximal remnant is sometimes termed the *lingual duct*.[9] By about 41 dpc (CS 17), the two lobes of the gland are seen to curve around the carotid arteries and are connected by the often rather delicate isthmus.

By about 44 dpc the lobes of the thyroid gland grow laterally and soon become closely associated with the *ultimobranchial bodies* (also occasionally termed the *telopharyngeal bodies*),[2] the nonparathyroid derivatives of the fourth pharyngeal pouches.[4,10] These then become surrounded by thyroid tissue and gradually become incorporated into it. It is by this means that the neural crest-derived parafollicular or C-cells become incorporated into the thyroid gland. By about 51–52 dpc, the thyroid exhibits its definitive form and is located anterior to the first six tracheal cartilages, although the isthmus is usually opposite the second and third tracheal rings. The thyroid follicles are first recognized during the early fetal period when the Crown-Rump (CR) length of the conceptus is about 40–50 mm. Colloid is first observed in the thyroid follicles in CR fetuses 60 mm in length, and the early colloid production period extends from about 65 mm to 80 mm.[11] With the differentiation of the gland, there is a substantial increase in its vascularization. It also develops a rich lymphatic system.

DEVELOPMENT OF THE THYROID GLAND IN THE HUMAN FETUS AND DURING THE PRE- AND EARLY POSTNATAL PERIOD

By contrast to the considerable number of articles written on the embryonic development of this gland, relatively little has been published on its subsequent prenatal and early postnatal development. Possibly one of the most comprehensive articles that considers the latter period was written by Weller, although the majority of that study is concerned with the events that occur during the embryonic period.[4] Two other papers also provide useful information on its features during the early fetal period. Aboul-Khair et al.,[12] for example, studied the development of the fetal thyroid gland after the administration of radioactive iodine to the mother at various times before the termination of pregnancy. They noted that the growth and iodine-trapping function of the fetal thyroid was age dependent up to the 23rd week of gestation while the biological half-life of the radioiodine was much shorter than that of the adult up to the 19th week of gestation.

Shepard[13] subsequently divided the early development of the thyroid gland into three stages. He termed these as follows:

1. The precolloid period (CR length 22–64 mm, approximate gestational age 47–72 dpc).
2. The beginning of the colloid stage (CR length 65–80 mm, approximate gestational age 73–80 dpc).
3. The stage of follicular growth (CR length greater than 80 mm, approximate gestational age greater than 80 dpc).

He indicated that by Stage 2, the thyroid gland achieved maturity in terms of its relative weight compared to that of the total body weight. This is very similar to that in the newborn and in the adult. Also, at about this time the histological appearance of colloid-containing central cavities and the ability to produce iodinated organic products including T4 is similar to that seen in the newborn. Shepard also noted that the central colloid cavities formed from the enlargement and fusion of the intracellular canaliculi and that the development of the latter was possibly controlled by the influence of thyroglobin.

Ultrastructural observations reveal that during the so-called precolloid period relatively little cytoplasm and endoplasmic reticulum are present in the thyroid epithelium. Similarly, the ribosomal particles are relatively unattached. During the early colloid period, there is an increase in rough endoplasmic reticulum; however, the Golgi apparatus, although present, is not particularly conspicuous. By about the 28 mm CR length stage (at about 56 dpc), the thyroid cells now contain intracellular cavities, and these are lined by microvilli. These later become more dilated and gradually move toward the apex of the cell. These spaces eventually open extracellularly and coalesce together. This appears to be the first evidence of colloid cavity formation. No similar sequence had been described in the rat during the embryonic period.[14] The earliest stage of human development when iodine was found to be concentrated was at about 74 days of gestation.[15]

HISTOLOGICAL AND ULTRASTRUCTURAL MORPHOLOGY OF THE THYROID GLAND IN THE HUMAN

At the ultrastructural level, it is possible to recognize two types of cells within the thyroid gland: (1) *principal cells*; and (2) *parafollicular cells* (also termed "light" or "clear" cells).[16] These two cell types are quite distinct. The principal (or epithelial) cells vary in their appearance according to whether the cell is at the *resting* (or synthetic) or *active* (or secretory) phase. In the former phase, the epithelial cells have either a squamous or low cuboidal appearance. When they are in the active phase, these cells tend to be columnar. The nucleus of these cells tends to be round or ovoid and possesses a limited amount of heterochromatin and contains only one or two nucleoli. The cytoplasm of these cells is basophilic. Toward their apices, in their synthetic phase, the cytoplasm contains large numbers of secretory droplets that are involved in the transfer of thyroglobulin into the colloid located within the follicle. These secretory droplets receive the thyroglobulin from the Golgi complexes that tend to be located close to the nucleus of the cell, and transport it for eventual exocytosis into the follicle.[17,18] The apical surface of the epithelial cells is covered with large numbers of short microvilli.

By contrast, the parafollicular cells tend to occur either singly or in small groups in proximity and are usually adherent to the basal region of the principal cells. They also tend to be substantially larger than the principal (endothelial) cells, often two to three times larger and are always separated from the lumen of the follicles by a continuous layer of endothelial cells. In the human, these represent only a very small proportion (about 0.1%) of the total thyroid cell population but have a quite distinct and characteristic morphology. While, like the endothelial cells, they possess a round or ovoid nucleus, their cytoplasm tends to contain a moderate amount of endoplasmic reticulum. Their characteristic feature of their cytoplasm, however, is the presence within it of very large numbers of electron-dense secretory granules that measure about 0.1–0.4 μm in diameter. The secretory granules of these cells contain *calcitonin* (or thyrocalcitonin),[19] whose principal function is to lower the circulating level of calcium in the plasma principally by suppressing bone resorption.[20,21]

While in the human calcitonin plays an important role in the control of calcium levels in the body, it appears to be less important in this regard than in many other mammalian species. In the dog, for example, Kameda[22] and others[23] noted a ratio of 30–90 parafollicular to 100 follicular cells. As noted elsewhere, these cells take origin from the ultimobranchial bodies, and when the latter become incorporated into the thyroid their parafollicular or C-cells soon become dispersed within the thyroid gland.[24,25] In some mammalian species, principally during the fetal and early postnatal period, these cells also contain *serotonin* (5-hydroxytryptamine), although its exact significance in these species remains to be established. It has been demonstrated that these cells also contain

somatostatin.[26,27] The latter substance inhibits the secretion of insulin and glucagon by the pancreas and growth hormone and thyroid-stimulating hormone (TSH) by the pituitary. While the exact role of somatostatin has yet to be established, it has been suggested that it may be involved in the local regulation of the secretion of calcitonin and/or thyroid hormone within the thyroid gland.

SITES OF ECTOPIC THYROID TISSUE

The presence of ectopic thyroid tissue is invariably of developmental origin and is usually found along the normal line of descent of the gland. This thyroid tissue may differentiate in close proximity to the foramen caecum, in the dorsal midline of the tongue at the junction between the anterior two thirds and the posterior one third of the tongue. Should thyroid tissue develop in this location, this gives rise to a *lingual thyroid*. Ectopic thyroid tissue may also be present within the tongue musculature. The thyroglossal duct may persist, and one or several cysts may develop from it along its path of descent. A *thyroglossal cyst* or nodule is usually present as an isolated entity and may occasionally be of substantial size. It is invariably located in the ventral midline and characteristically moves rostrally and caudally on swallowing. Rarely, a *thyroglossal sinus* may be present and may communicate with the surface of the neck. Should the most caudal part of the thyroglossal duct persist, this may give rise to a *pyramidal lobe* or lobule of the thyroid gland. It ascends toward the hyoid bone and is usually attached either to the isthmus region of the gland or to the adjacent part of one or other lobe (more often the left than the right). It is relatively commonly encountered, and possibly seen in about one third of humans. It may be detached from the gland and may even be present in two or more parts.[28] Occasionally, the thyroid isthmus may be absent. Similarly, the thyroid gland may fail to descend to its normal location. In most cases in which the latter condition is observed, this is not associated with abnormalities of thyroid functioning.

A very rarely encountered condition that may be explained along these lines is *struma intralaryngotrachealis*. In this condition, thyroid tissue may be found *inside* the larynx and trachea, although Boyd has illustrated a histological section of the neck region of a 145 mm CR length human fetus that displays an aberrant nodule of thyroid tissue that lies between the thyroid and cricoid cartilages. The author has suggested that this might have given rise to a nodule of thyroid tissue located within the trachea had this fetus survived.[8] It is also worth noting that cystic remnants of the ultimobranchial body may be found within the adult thyroid gland. These are recognized by the presence of ciliated epithelial cells.

DEVELOPMENT OF THE THYROID GLAND IN THE MOUSE

The histologic development of the mouse thyroid gland is illustrated in Figures 4.1 through 4.6. These virtual slides can be scanned and further magnified and are available on the accompanying DVD.

According to Theiler,[29] the thyroid gland is the first of the endocrine organs to develop in the mouse. Its presence is first noted on about 8.5 dpc in embryos with about eight pairs of somites (at about Theiler Stage [TS] 13) as an endodermal thickening in the floor of the pharynx. At this stage, the thyroid primordium is both dorsal and in close proximity to the aortic sac. Shortly afterward (TS 15–17), it shows evidence of differentiation and, like the human gland, first appears as a hollow diverticulum located in the ventral midline between the first and second branchial arches. In sagittal sections, it is seen to be located just rostral to the heart rudiment, at about the level of the truncus arteriosus/aortic sac. By TS 17–18, the thyroid primordium is hollow and shaped like a flask and possesses a thick basal wall. It is also, like the situation observed in the human, initially attached to the floor of the pharynx by a narrow stalk, termed the thyroglossal duct. The latter soon involutes (TS 18–19), and the glandular caudal component descends in the anterior part of the neck and loses its lumen. By TS 20, the thyroid has descended to the level of the arch of the aorta, while its rostral connection with the pharynx, at the foramen caecum, has almost disappeared. The thyroid gland at this stage is solid.[30] Around this time the cells of the thyroid become eosinophilic and may appear to be in the form of rosettes.

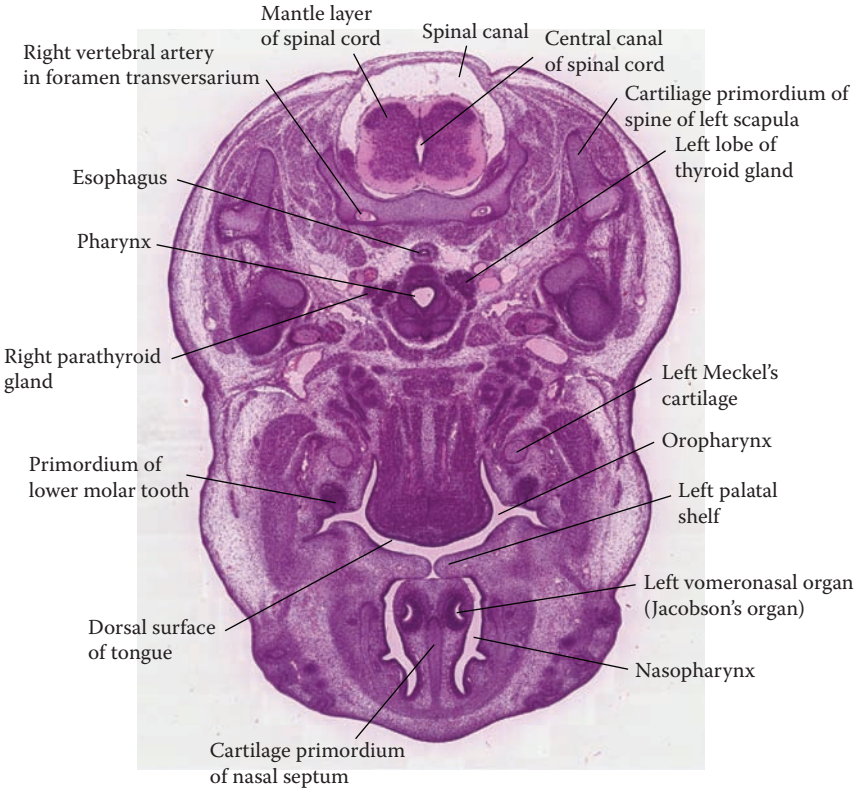

FIGURE 4.1 (E14.5_Fig4.1.svs) Low magnification, transverse section through the rostral part of the pharynx and base of the tongue of a mouse embryo (E14.5) stained with hematoxylin and eosin (H&E). It is possible to recognize the left lobe of the thyroid gland and right parathyroid gland in this section.

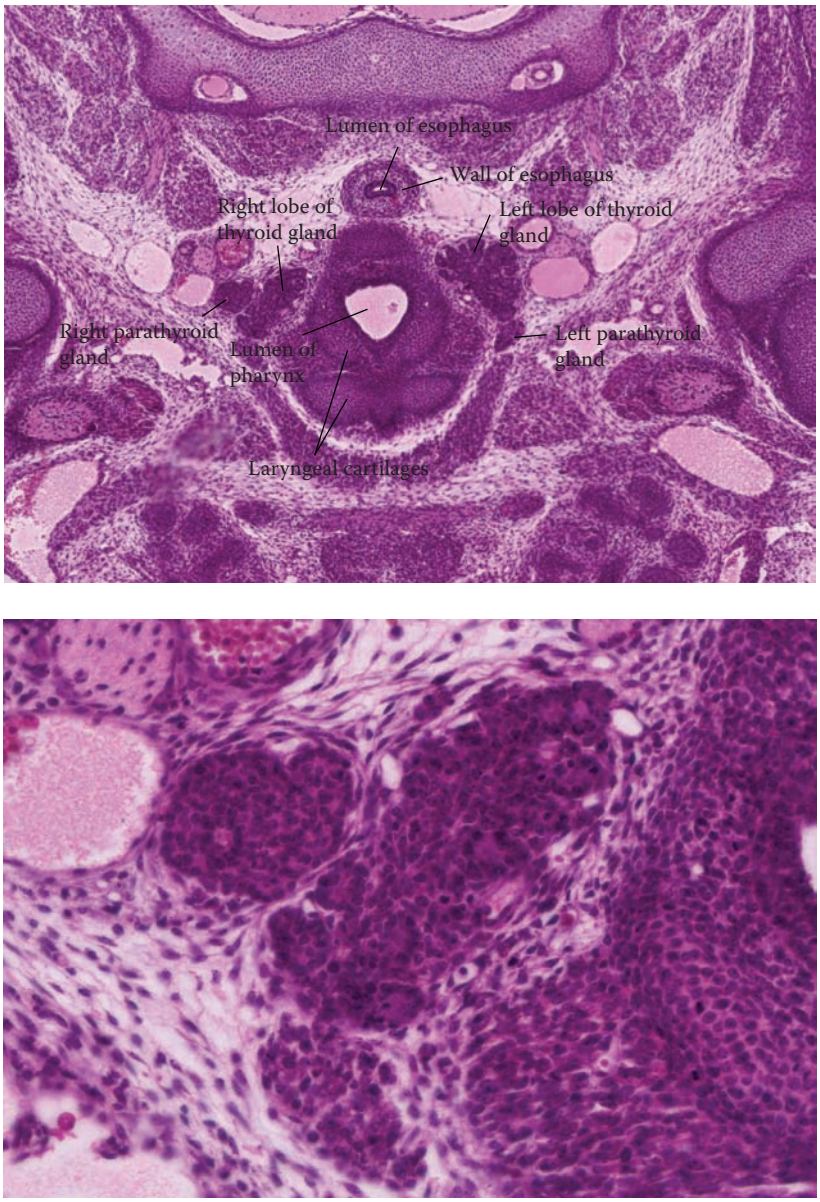

FIGURE 4.2 Medium (top) and high magnification of Figure 4.1. These illustrate the left and right lobes of the thyroid gland and the right parathyroid gland in addition to the laryngeal cartilages and the lumen of the pharynx. It is also just possible to recognize the left parathyroid gland. High magnification image (bottom) shows right lobe of the thyroid gland and right parathyroid gland.

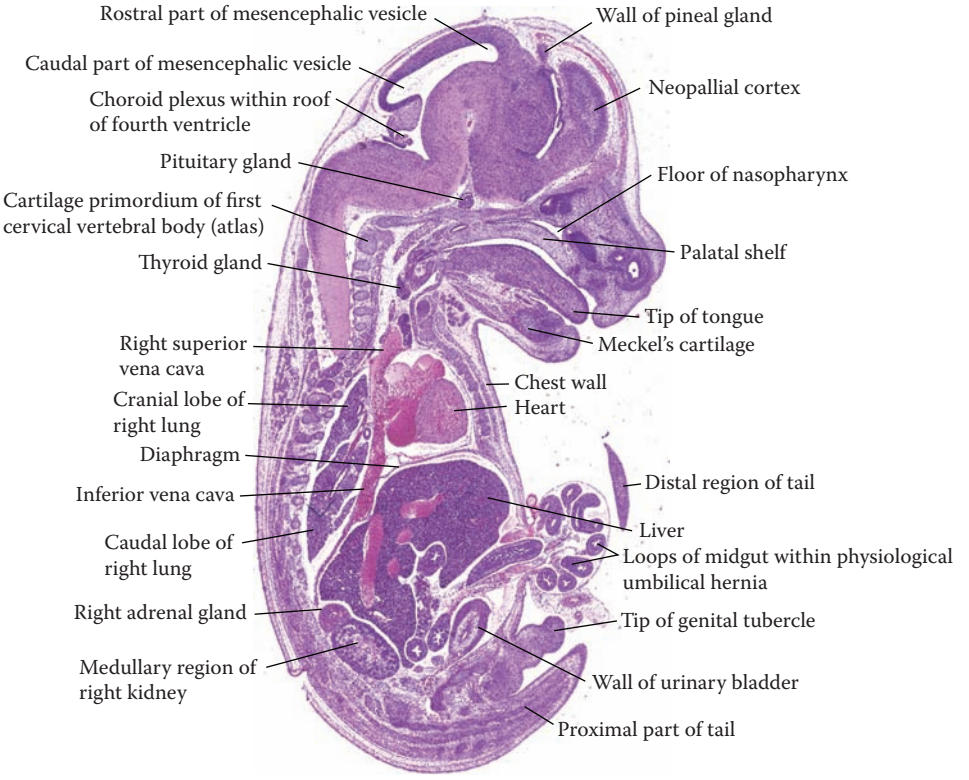

FIGURE 4.3 (E15.5_Fig4.3.svs) Low magnification, right sagittal section through the neck region of a mouse embryo (E15.5–16) stained with H&E. It is just possible to recognize the right lobe of the thyroid gland in this section.

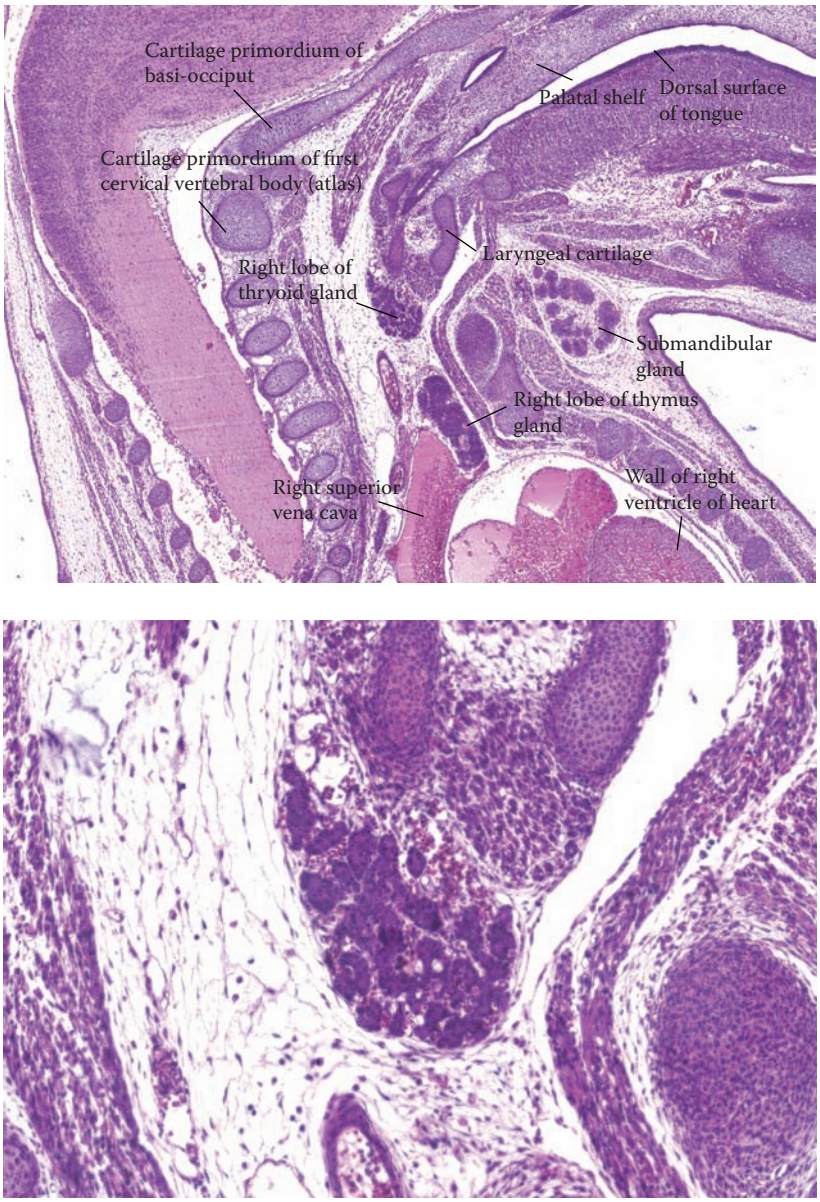

FIGURE 4.4 Medium (top) and high (bottom) magnification of Figure 4.3. These show the right lobe of the thyroid gland. This is closely associated with components of the right part of the laryngeal cartilage. Its proximity to the cartilage primordial of the cervical vertebral bodies is also clearly seen in the medium magnification image.

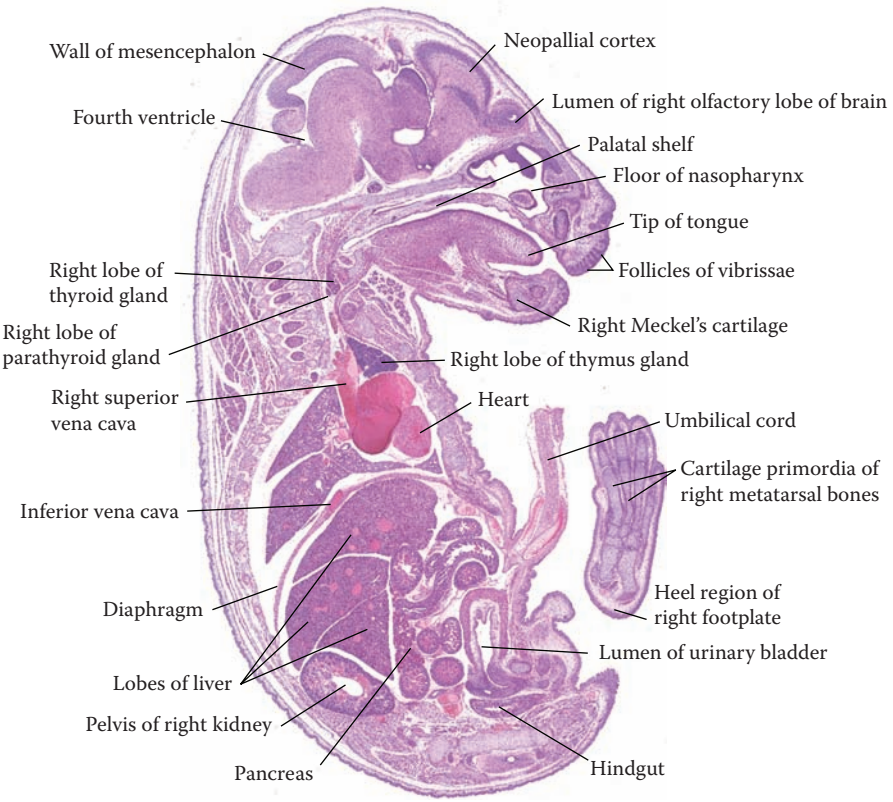

FIGURE 4.5 (E17.5_Fig4.5.svs) Low magnification, right sagittal section through the neck region of a mouse embryo (E17.5–18) stained with H&E. It is just possible to recognize the right lobes of the thyroid and parathyroid glands in this section.

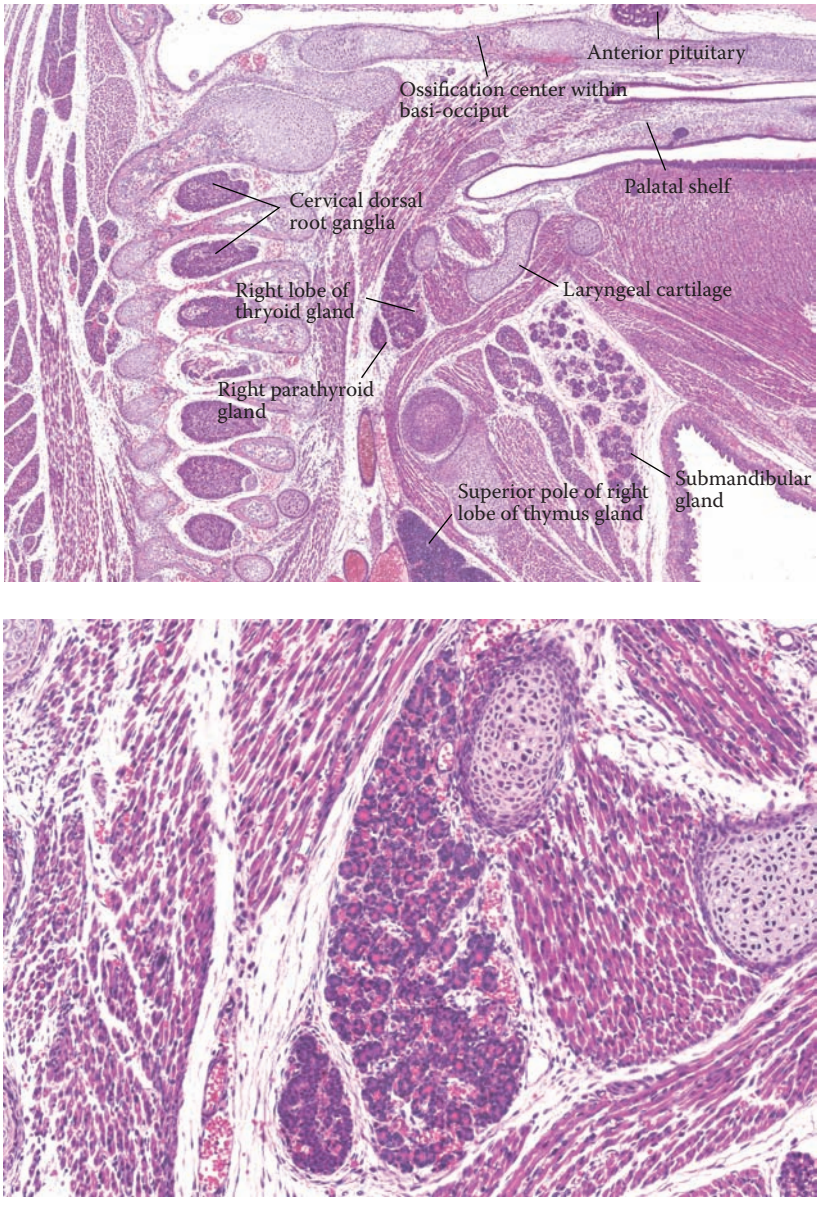

FIGURE 4.6 Medium (top) and high (bottom) magnification of Figure 4.5. These demonstrate the right lobes of the thyroid and parathyroid glands and their close association with the right parts of the cervical vertebral bodies and right dorsal root ganglia (medium magnification image).

Fusion soon occurs between the lateral parts of the lobes of the thyroid and the ultimobranchial bodies, the derivatives of the caudal parts of the fourth and possibly fifth pharyngeal pouches. As a result, the neural crest-derived parafollicular or C-cells become incorporated into the thyroid gland. The single pair of parathyroid glands seen in the mouse also becomes closely associated with the thyroid gland.[29,31]

By 15 dpc (TS 23), the thyroid gland is divided into small buds separated by blood vessels, although follicles are not yet seen at this stage. The parathyroids are, however, readily distinguished from the thyroid gland at this stage. By about 17 dpc (TS 25) the thyroid is now a bilobed structure containing solid branched epithelial cords and early evidence of well-vascularized follicles. It has been suggested that there is some evidence of secretory activity from about 16 to 17 dpc.[30] Large numbers of colloid filled follicles are seen in the gland on or about 18 dpc (TS 26). Between the lobules, the loose connective tissue contains large numbers of blood and lymphatic vessels, and there is now considerable evidence of secretory activity.

HISTOLOGICAL FEATURES OF THYROID GLAND DEVELOPMENT IN THE ADULT MOUSE

By birth, all of the gross anatomical adult features are present. Thus, the thyroid now possesses two elongated lobes, and these are joined across the ventral midline of the trachea by a thin isthmus. The lobes of the thyroid gland are also by now deeply embedded within the muscles of the neck and are particularly well vascularized. (The pretracheal fascia separates the gland from a number of muscles. The gland is covered antero-laterally by the *sternothyroid* muscle, while the *sterno-hyoid* muscle is more superficially located than the sternothyroid muscle.) While in the mouse the rostro-caudal extent of the gland is over the first three or four tracheal rings, in the rat the lobes often lie ventrolateral to the first four or five tracheal rings. In both of these species, their location and size often vary to some degree.

The size of the thyroid follicles depends to a considerable degree on the age of the individual, its diet, endocrine status (whether they are at the synthesis or secretory phase), and the sex of the individual. In young mice they tend to be uniform in size, spherical in shape, and lined by columnar cells with spherical nuclei. The colloid is also homogeneous and *slightly eosinophilic*, and the colloid present within them tends to be semifluid in consistency and is normally in contact with the follicular epithelium. During the early postnatal period (aged 1–3 months), the height of the epithelial cells decreases and follicular diameter increases.[32] In *inactive* glands the colloid tends to be eosinophilic, while in *active* glands it is principally basophilic. The thyroid also possesses C-cells of ultimobranchial body origin. These cells possess calcitonin-containing granules. Although they do not stain well with hematoxylin and eosin (H&E), such granules stain intensely with silver staining techniques due to their catecholamine content. They may also be stained using appropriate immunocytochemical methods. Most of these cells tend to be located toward the center of the gland, while there may be no C-cells toward the periphery of the gland.[33]

In one of the only studies undertaken to determine the rate of growth of the thyroid gland, Wechbanjong et al.[34] established that the volume of the gland in Swiss Webster mice increased dramatically over the period between birth and 8 weeks of age. They calculated that at 8 weeks of age, the thyroid gland was 14-, 4-, and 3-fold greater than the volume in the 1-day-old, 2-week-old, and 4-week-old mouse. They also established that the average number of C-cells present in the gland at 8 weeks was 18-fold, 5.5-fold, and 2.5-fold greater than the number observed in the thyroid at 1 day, 2 weeks, and 4 weeks of age, respectively. The concentration of C-cells was, however, greatest in the 4-week-old mice. C-cells were seen in mitosis in mice up to 4 weeks of age but not in the 8-week-old mice. At all ages studied, the C-cells contained calcitonin.

It has also been noted that with increasing age the follicles become larger and more variable in size and that the interfollicular connective tissue in the gland decreases. The colloid within them

also becomes *more eosinophilic*. In older mice, particularly females, two types of follicles are recognized. Just over half are *inactive* follicles with flattened epithelium and increased amounts of colloid, and these appear to coexist in the gland with smaller *active* follicles with cuboidal or columnar epithelium lined by microvilli. In the active cells, the cytoplasm contains lysosomes that increase in number with age. Follicular size varies considerably, and some of the larger follicles merge together to form bilocular or trilocular cysts. These generally possess a flattened epithelium.[35] In addition to the follicular and parafolliciular cells that have long been observed in the thyroid gland, the other cells, possibly also of ultimobranchial origin, have also been reported in the rodent thyroid gland.[36]

THE THYROID GLAND IN THE ADULT RAT

According to Hebel and Stromberg,[37] in the adult rat the two thyroid lobes are rhomboidal in shape and measure 3.9–5.5 mm in length and 2.0–3.0 mm in width. The average weight of the entire gland is said to be about 15.4 mg in the male and 17.9 mg in the female. These weights are quite different from those reported previously by Hatai,[38,39] who indicated that in the male the entire gland weighed about 34.0 mg, and in the female about 32.0 mg. By contrast, Remington et al.[40] calculated that the weight of the entire gland was related to that of the adult body weight, being about 7.5 mg/100 g body weight. Remington et al. indicated that the weight of the thyroids in their study were less than that observed previously. They suggested that any difference in size might have been due to the amount of iodine in the diet. The weights of the thyroid also appeared to be very strain specific. For example, see the weights of this organ as determined by Freudenberger.[41]

The thyroid gland is surrounded by a thin capsule; while it largely consists of collagenous tissue, the gland also contains adipose tissue and sinusoidal blood vessels that are continuous with those in the stroma of the gland. The follicles within the gland are mostly separated by connective tissue septae. The larger follicles tend to be spherical in shape, with the largest follicles measuring up to 270 μm in diameter. The smallest follicles tend to be centrally located and measure up to about 120 μm in diameter. The isthmus of the gland, when present, contains vascular elements and a few isolated follicles.[37,42]

It is relevant to note that the histological morphology of the follicular epithelium depends on the functional state of the follicle. In resting (i.e., synthesizing) cells, the epithelium is cuboidal (up to about 4 μm), while in active (i.e., in secretory cells) it is usually columnar (up to about 10 μm). The colloid is also more acidophilic in the resting follicles than in the active follicles. A number of studies have been published on the anatomical basis of secretion of the follicular cells in the rat.[43,44] Within the stroma, there are large numbers of parafollicular or C-cells.[45] These are usually located between the epithelial cells or sometimes within the lumen of the follicle.[37,46] However, they are more frequently located between the basement membrane and the follicular epithelium and not in contact with the colloid. It has been noted above in relation to the situation in mice that the C-cells usually occupy an elongated region in the central part of the lobe and decrease in number toward the periphery of the lobe, where no C-cells are usually found.[33] The area occupied by the C-cells, and their total number is greater in the autumn than in the spring.[47]

CONTROL OF THYROID DEVELOPMENT IN MICE

Over the last 10 or so years, an enormous number of articles have been published describing studies using molecular methodology to investigate principally the genetic factors believed to influence the early development of the thyroid gland. In almost all these studies, the mouse was selected as the experimental animal of choice. Other studies are also described in which mouse models of certain relatively common thyroid diseases were prepared to allow the factors that induce these conditions to be investigated and strategies for treating these conditions to be developed. The articles described in this section can therefore be representative only of the range of studies undertaken

during this period. An approximately chronological approach has been taken here, as this reflects to a very general degree the advances in the methodology that have been applied to investigating these problems.

In a relatively early paper of the type indicated above, the role of insulin-like growth factor 1 (IGF1) on follicular cell growth response *in vitro* to TSH was studied.[48] IGF1 mRNA localization was investigated using the technique of *in situ* hybridization in tissue sections of the mouse thyroid gland, and the thyroid IGF1 mRNA was reported to be principally found in follicular and C-cells rather than in the stroma. Moreover, levels were higher during postnatal thyroid growth and following gland stimulation with TSH. Thomas et al. did note, however, that the level decreased in mature animals, although this was principally due to the reduction in the follicular cells, as it was still easily demonstrable in the C-cells and in the parathyroids. It was believed that the follicular cells were the main source of IGF1 in the thyroid, and they concluded that the role of IGF1 in follicular cell growth was as an autocrine factor.

In a study undertaken to investigate the influence of the *Hoxa3* gene on the *migration patterns* of labeled mesenchymal neural crest into the thymus and thyroid gland in mutant embryos, only a minimal effect was noted.[49] It was believed that the loss of this gene affects the intrinsic capacity of mesenchymal neural crest to differentiate and/or induce proper differentiation of the surrounding pharyngeal arch and pouch tissues, as *Hoxa3* homozygous mutant mice are athymic and display thyroid hypoplasia. The thyroid differentiates from two sources—the thyroid diverticulum and the ultimobranchial body—both of which express *Hoxa3* and are defective in these mutant mice.

On occasions, it was noted that a vesicle was present that was composed exclusively of calcitonin-producing cells and suggested that this represented the persistence of the ultimobranchial body. A particularly confusing feature was that variable expressivity was noted among the mutant mice and even between the two sides in the same animal. They therefore suggested that this might reflect the presence of a compensatory gene or genes and proposed that this might be the paralogous gene *Hoxb3*.

Mice were generated that lacked the thyroid-specific enhancer-binding protein (T/ebp, currently called the NK2 homeobox 1[*Nkx2-1*]).[50] This produced mice that lacked the expression of T/ebp, a transcription factor known to control thyroid-specific gene transcription. While heterozygous mice were normal, the homozygous mutants were born dead and lacked lung parenchyma, although they possessed a rudimentary bronchial tree with abnormal epithelium. The homozygous mice also possessed no thyroid gland but had normal parathyroids. Extensive brain defects were also present, particularly involving the ventral forebrain, and the pituitary was always completely absent. Appropriate studies indicated that the T/ebp gene was expressed in the normal thyroid, lung bronchial epithelium, and specific areas of the forebrain during embryogenesis. This study indicated that the expression of T/ebp was also essential for the development of the thyroid, lung, ventral forebrain, and the pituitary.

A study was undertaken to examine, on day 18 of gestation, the thyroid gland of athymic nude mice ($Foxn1^{nu}/Foxn1^{nu}$), and its morphology was then compared with that of control littermates ($Foxn1^{nu}/+$) at a similar stage of gestation.[51] In the nude mice the epithelial cell height and diameter of the thyroid follicles were significantly smaller than in the controls, and fewer well-developed follicles were located at the periphery of the gland than in the controls. The weight and total volume of the thyroid was also smaller. In a complementary ultrastructural study of the thyroid gland of these homozygous mutant and control littermates, the areas of the epithelial cells, their nuclei, cytoplasm, and mitochondria were measured.[52] While the areas of the nuclei were similar, in all other regards the areas of these cells and most of their components were smaller in the mutants than in the controls. It was suggested that the thyroid glands were underdeveloped at the end of gestation compared to the situation observed in their normal littermates.

In another study, a mouse model showing many of the similarities to the human condition of *hereditary thyroid dysgenesis* was induced.[53] In the 1:3,000–4,000 infants born with hypothyroidism,

80% have either an ectopic thyroid, small sublingual thyroid, or no thyroid tissue present. Most of the clinical consequences of hereditary hypothyroidism are now eliminated by appropriate treatment. The location of the gene designated *Titf2* (current designation: *Foxe1*, forkhead box E1 (thyroid transcription factor 2)) is on mouse Chromosome 4. This is expressed in the developing thyroid, in most of the foregut endoderm, and in the craniofacial ectoderm, including that of Rathke's pouch (RP). When the expression of the gene precursors is down-regulated, the thyroid cells cease migration, suggesting that this factor is involved in thyroid morphogenesis. It was also noted that these homozygous mutant mice developed a cleft palate and either possessed a sublingual thyroid or were born with a completely absent thyroid gland.

An attempt was made to establish a mouse model for thyroid carcinogenesis that was also associated with the production of metastases,[54] as this would allow the possibility of understanding the molecular basis for this condition. Such a mutant mouse was established (termed the TRbetaPV mouse). In this mouse, the thyroid-pituitary axis was disrupted. This led to a mouse with high levels of circulating TSH, which was associated with hyperplasia of the follicular epithelium within the thyroid. In the aged homozygous mutant mice, thyroid carcinoma metastases developed. Histological evaluation of the thyroids in these mice displayed a high incidence of capsular invasion, vascular invasion, anaplasia, and metastases to the lung and heart. This model allowed the possibility of studying the alterations that occur in gene regulation during thyroid carcinoma development and its metastatic dissemination.

Primary cell cultures of mouse thyroids were established that displayed strong cytoplasmic staining for thyroglobulin for up to 14 days after seeding.[55] After 28 days, only 5–10% of the thyrocytes displayed these staining properties. The cultured thyrocytes expressed the thyroperoxidase and thyrotropin-receptor genes and, although at lower levels, the sodium iodide symporter gene. The cultured thyrocytes could also be transfected using the FuGENE 6. It was suggested that the production of these cultured thyrocytes could now be used for a variety of studies particularly within the field of thyroid pathophysiology.

To assess any possible damage among increased or decreased metabolic rate, oxidative stress, and oxidative damage, female mice were rendered either hyperthyroid or hypothyroid by the chronic administration of either L-thyroxine or thiouracil, respectively, in their drinking water over a period of 5 weeks.[56] Hyperthyroidism significantly increased the sensitivity to lipid peroxidation in the heart, although the endogenous levels of lipid peroxidation were not altered. Hyperthyroidism also induced oxidative damage to mitochondrial DNA and to a lesser extent genomic DNA. Hypothyroidism, by contrast, decreased damage to mitochondrial DNA. The results indicated that thyroid hormones modulate damage to lipids and DNA in the mouse heart. Treatment with a thyroid hormone inhibitor reduced oxidative damage in the various cell compartments studied.

While it was known that thyroid hormone (T3) affected epithelial cell differentiation during lung development in the late fetal period, its affect on earlier stages of lung development were unknown. It was hypothesized that T3 would alter embryonic lung airway branching and other aspects of early lung morphogenesis. To test this hypothesis, mouse lungs were isolated at 11.5 dpc and maintained in tissue culture (TC) for 72 hours either without or in the presence of increasing doses of T3 in the TC medium.[57] In the presence of T3 a dose- and time-dependent decrease in branching, morphogenesis was observed. When lungs were cultured in higher doses of T3, cell proliferation and mesenchyme formation were significantly *decreased*, while an *increase* in cuboidal epithelium and airway space resulted. Consequently, it was demonstrated that during the early stages of lung development, accelerated epithelial cell and mesenchymal cell differentiation occurred at the expense of new branch formation and lung growth.

More recent studies involving the use of appropriate transgenic mice have provided insight into the mode of action of thyroid hormone. In a series of targeted mutagenesis (so-called knockout) experiments involving mice, one or more of the thyroid hormone receptor isoforms were deleted. One of these isoforms (termed TR-beta2) is of particular importance in that it mediates negative feedback control of the hypothalamic-pituitary-thyroid axis. Analysis of these targeted

mutant (knockout) mice revealed that they exhibited a much milder phenotype than hypothyroid animals. Animals expressing a complete hypothyroid phenotype were subsequently produced, and it was possible to demonstrate that the unliganded thyroid hormone receptors mediated the effect of hypothyroidism.

It had long been established that TSH is the principal agent involved in the regulation of the thyroid gland. This hormone acts by binding to the TSH receptor (TSHR) and then triggering the activation of the adenyl cyclase pathway. This regulates both proliferation and functioning of the adult thyroid cells. De Felice et al.[58] found that in their rodent model, TSH did not appear to control the growth of the thyroid during the fetal period as it does in the adult. A mouse phenotype was also studied that was deficient for TSH-TSHR signaling. Patients with congenital hypothyroidism due to the loss of function mutations in the receptor gene have been studied.

In another recent study, a mouse model for the relatively common human condition of autoimmune (lymphocytic) thyroid disease (also termed autoimmune thyroiditis) was described.[59] It had formerly been observed that thyroid peroxidase was a well-characterized autoantigen in this condition and that autoantibodies and autoreactive T lymphocytes in particular played a major role in this disease. To understand the underlying mechanism, and particularly the role of thyroid peroxidase, a mouse model of this condition was induced. Antibodies against recombinant mouse thyroid peroxidase were detected in the serum of all of the treated mice after day 21, and Ng et al.[59] noted that the effect produced was dose dependent. All of the induced mice developed thyroiditis with associated destruction of their follicles, as characteristically seen in this disease. No similar effect was seen in any of their control mice. It was suggested that this model allowed the possibility of developing new strategies for modifying the abnormal immune response seen in these individuals.

A number of mouse models for human medullary thyroid cancer were produced.[60–64] At the time of this writing, tumors in humans can be treated only by radical surgery, as such tumors are resistant to both radiotherapy and chemotherapy. The human tumor is a C-cell neoplasm that characteristically secretes calcitonin. As such tumors also produce high levels of prostaglandins, nonsteroidal anti-inflammatory drugs such as indomethacin have also been employed to treat them.[60] Accordingly, the effect of indomethacin was tested in a nude mouse model of this condition and produced a dramatic inhibitory effect on tumor growth, principally by decreasing the number of proliferating cells within the tumor. It had also been noted that the expression of 15-prostaglandin dehydrogenase was increased in the treated tumors. Such preliminary results confirm the potential for this form of treatment in this condition by its action on one of the factors involved in its development, even if only as a precursor treatment to reduce tumor size before surgery.

Thyroid dysgenesis is encountered in a high proportion of human patients with congenital hypothyroidism. In a study to investigate this topic, a possible role of Sonic hedgehog (*Shh*) was noted as a regulator of thyroid development.[65] In *Shh* targeted mutant mice, while the thyroid primordium development was initiated, its budding was delayed, and at later stages in its development the thyroid failed to form a bilobed structure. In these mice, the single lobe that forms usually develops on the left side and appears to function normally. Aberrant thyroid tissue commonly develops in association with the trachea in these mice, particularly into the adjacent mesenchyme and occasionally into the tracheal lumen. These ectopic growths form follicle-like structures that accumulate thyroglobulin. These studies appear to indicate that *Shh* plays a role in governing the formation of the two lobes of the thyroid gland during its late organogenesis, and it has been suggested that it may also play a part in inhibiting the formation of ectopic thyroid tissue. These observations are consistent with reports of other situs anomalies in *Shh*-deficient mice.[67] It is well known that *Shh* in the chick is expressed in an asymmetric fashion on the left side of Hensen's node.[66] This strongly implies that this gene is a *left determinant* in this species. By contrast, in the mouse, *Shh* is expressed *in the midline* and is required to prevent left determinants from being expressed on the right.[67,68]

THE PHYSIOLOGICAL FUNCTIONS OF THE THYROID GLAND

As with the numerous published articles on development and histological morphology as well as on the anatomical features of the thyroid gland cited elsewhere in this chapter, much has been written on the normal physiological functions of the thyroid gland.[69] Similarly, much has been written on the various disease states to which it is liable, such as those associated with its underactivity (i.e., hypothyroidism), overactivity (i.e., hyperthyroidism), and the various malignancies to which this gland is subject. Equally, problems may be encountered if the gland is located ectopically. For these reasons, only a very brief overview of its principal physiological functions is provided here. Readers who might be particularly interested in these aspects of its activity will find little difficulty seeking out information about its various roles during the postnatal period and in the adult.

The thyroid gland weighs about 20–25 g in the adult human male, but it is usually slightly heavier in the adult female. It is also now possible to estimate the size of the thyroid gland noninvasively by using diagnostic ultrasound. Similarly, it is now possible to compare its volume with body weight, age, and sex in normal individuals.[70] These parameters have also been determined in 300 healthy children.[71] In the latter study, no significant difference was observed between males and females in each height group studied. The gland is nearly always asymmetrical, with the right lobe often twice the size of the left lobe. The gland enlarges during puberty, the menstrual cycle,[72] and pregnancy and is believed to change in weight according to the seasons, with a decrease in its mass occurring during the winter.

The thyroid gland has two principal roles. The first is to control the basal metabolism of the body and to maintain the growth of the individual during the preadult period. These activities are mediated through the production within the gland of a number of hormones and their secretion into the circulation as and when appropriate. The hypothalamo-pituitary-thyroid axis controls production of these hormones. Thyroid function is controlled by the production of TSH.[73] This is a glycoprotein secreted by the *thyrotroph* cells of the anterior pituitary in response to *thyrotropin-releasing hormone* (TRH) secreted by the hypothalamus. The whole system is under the feedback control of the thyroid hormones T3 and T4. A low level of circulating hormones causes an increase in the secretion of TSH, whereas an increased level of circulating thyroid hormones causes a decrease in its secretion.

As mentioned previously, one of the unique features of the thyroid gland is that it is able to store large amounts of these hormones within the *colloid* located in its extracellular follicles. The iodine ingested in the diet passes to the gland via the bloodstream, and here it is concentrated. The colloid stores an iodinated glycoprotein, iodothyroglobulin, which is a product of the follicular epithelial cells and the precursor of T3 and T4. A substantial plexus of fenestrated capillaries and an extensive network of lymphatic vessels surround the follicles. While a dense network of sympathetic nerves supplies the capillaries and their feeder vessels, some may also supply the follicular epithelial cells.[74,75] When released into the bloodstream, the hormones are bound to proteins, and only a relatively small proportion circulates as free hormone. The majority of the T4 is converted by the target cells to the more biologically potent T3, most of which is obtained by this route as opposed to direct production by the thyroid gland. Thus, T4 acts primarily as a prohormone for T3.

If an individual enters an iodine-deficient region of the world, these systems are capable of maintaining them in a euthyroid state over a lengthy period of time. Should, however, there be a prolonged restriction in the dietary intake of iodine, the thyroid gland usually undergoes compensatory enlargement to form a *goiter* (also termed a colloid goiter).[76] In the newborn, congenital hypothyroidism may be associated with severe mental retardation if not appropriately treated at an early stage. By contrast, antithyroid autoantibodies are associated with certain forms of hypothyroidism termed autoimmune thyroiditis. One of the more common manifestations of hyperthyroidism is Graves' disease, and this is often associated with ocular manifestations, termed exophthalmos.[77]

The second role of the thyroid gland is to maintain calcium homeostasis. This is achieved by controlling the plasma calcium level within relatively narrow limits by secreting *calcitonin*. This

hormone is secreted by the parafollicular or C-cells (also termed "light" or "clear" cells). As previously stated, they are of neural crest origin and incorporated into the thyroid gland when it amalgamates with the ultimobranchial body.[78] For details on the role of the parathyroid glands and calcitonin in maintaining calcium homeostasis in the body, see Chapter 5 in this volume.

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5 The Parathyroid Gland

INTRODUCTION

Relatively little has been written recently on the development of the two pairs of parathyroid glands that arise in the human and even less on the single pair of parathyroid glands that develop in the mouse. Those in the human are termed the parathyroid IIIs and parathyroid IVs because of their embryological origin from the dorsal part of the third and fourth pharyngeal pouches, respectively, while the single pair that develops in the mouse is exclusively derived from the dorsal part of the third pharyngeal pouch. With regard to the terminology of the glands in the human, because of the eventual location of these two pairs of glands, the parathyroid IIIs are often termed the *inferior* parathyroid glands whereas the parathyroid IVs are often termed the *superior* parathyroid glands. This is because of their usual eventual location in the neck region in the adult, as postero-medial relations of the thyroid gland. Because of the differences observed in the parathyroid glands in the human and in the mouse, it is appropriate to draw attention to what is known of their different developmental origin. As most workers in this field are likely to be aware of the situation in the human, the embryological origin in this species is considered first. It is of considerable clinical importance to be aware that as the thymus gland develops from the *ventral* part of the third pharyngeal pouch, the parathyroid glands that develop from the dorsal part of the third pouch may be located in a number of aberrant sites. This is of considerable importance when it is necessary to surgically remove a parathyroid tumor that has been diagnosed clinically, particularly if it is of the inferior gland and of third pharyngeal arch origin, as its location may be quite variable. It may be necessary initially to explore the neck region in the proximity of the thyroid gland and then progress caudally until finally the superior mediastinum is explored and the tumor is eventually located. The most caudal of these aberrant sites is in the superior mediastinum. Accordingly, if the parathyroid glands that develop from the third pouch retain their association with the thymic rudiment from the same side, they may also be located anywhere along the path of descent of the thymus gland or, for various reasons, elsewhere (see below).

ORIGIN OF THE STRUCTURES DERIVED FROM THE HUMAN THIRD PHARYGEAL POUCHES

The endodermally derived third pharyngeal pouch in the human is first clearly recognized in embryos with about 10 pairs of somites on about 23 days *post coitum* (dpc), which corresponds to about Carnegie Stage (CS) 10. Soon afterward, this region of the pharyngeal pouch makes contact with the ectodermally derived third pharyngeal cleft. As embryonic development progresses, the gap between the pouch and cleft narrows dramatically.[1] Contact with the surface ectoderm is, however, soon lost, and the ventral part of the pouch grows in a ventro-medial-caudal direction to give rise initially to the thymic rudiment. In the human, it has been suggested that there is no contribution to the latter from the surface ectoderm, although in other species it has been suggested that the definitive thymus has either an ectodermal component or may indeed be completely ectodermally derived. For example, in the pig, an ectodermal component from the third cleft contributes to the thymic rudiment, while in certain marsupials, it is believed that the thymic rudiment is exclusively of ectodermal origin. At about the 8–10 mm Crown-Rump (CR) length stage (at about 35–37 dpc, CS 15–16) the pharyngeal pouch is thick walled and possesses a dorso-cranial bulbous portion and

a caudal narrower and less bulbous portion. At this stage both the dorso-cranial and caudal portions of the third pharyngeal pouch are still attached to the pharynx by the *ductus pharyngo-branchialis III*. It is at about this stage that the antero-lateral wall of the dorsal bulbous portion begins to differentiate into the primordium of one of the parathyroid IIIs. At the same time, the remainder of the third pouch (i.e., its ventral part) of the same side gives rise to the corresponding half of the thymic rudiment. Shortly afterward, at about 40 dpc in about the 13 mm CR length stage embryo, the *ductus pharyngo-branchialis III* becomes a solid structure. When this occurs, the ductus pharyngo-branchialis III or IV in the human sometimes fragments and may develop into a series of irregular fragments. Some of these give rise to accessory parathyoid or thymic rudiments or may give rise to epithelial cystic structures. After the solid structure forms, it gradually separates the third pouch derivatives from the pharyngeal wall.

By about the 16 mm CR length stage (at about 44 dpc), Weller[2] calculated that the size of the ventral pouch is about six times greater than it was at the 14 mm CR length stage (at about 37–41 dpc). This dramatic increase in its size during this relatively brief period is principally due to the change in the overall shape of the thymic rudiment and is also associated with its caudal and medial movement. It has been suggested that this increase in the size of the ventral part of the pouch is principally due to differential growth of the various parts of the thymic primordium.[3]

When the ventral part of the third pouch separates from the pharyngeal wall, the tissue derived from this pouch tends to grow at a considerable rate, and its lumen soon becomes obliterated. As development proceeds, its caudal pole also tends to grow toward the ventral midline. More particularly, as the most caudal part grows toward the ventral midline, its rostral connection with the pharynx is eventually lost. Under normal circumstances, the dorsal portion of the pouch destined to give rise to the parathyroid primordium is initially attached to, and descends with, the thymic rudiment. At about the 20 mm CR length stage (at about 47–48 dpc, or at about CS 19) the parathyroid IIIs usually separate from the thymic rudiment and then become associated with the postero-medial aspect of the thyroid gland. (For the normal sequence of events associated with the early stages in the development of the thyroid gland, see Chapter 4 in this volume.) For this reason, the parathyroid III primordia are almost invariably located more caudally than the parathyroid primordia derived from the dorsal part of the fourth pharyngeal pouches. In addition, because of this occasionally the parathyroid III primordia may retain their connection with the thymic rudiment and descend caudally with it, sometimes as far caudally as the superior mediastinum. If separation is delayed, then the parathyroid III gland descends with the thymic rudiment, as occasionally occurs and has been noted during parathyroid tumor removal.[4]

The eventual location of the parathyroid IIIs tends to be quite variable, and while they are usually found within the fascial sheath of the thyroid gland just caudal to the parathyroid IVs and close to the inferior pole of the thyroid gland, they are not infrequently located elsewhere. If, for reasons that are not entirely clear, they are located outside the fascial sheath, they may be found immediately *rostral to* the inferior thyroid artery. In either case, this vessel usually supplies the parathyroid III. For the reasons indicated above, one or occasionally both of the parathyroid IIIs may descend along with the thymic primordium into the thorax, even as far caudal as close to the surface of the pericardium. By contrast, they may not descend at all, and very occasionally one or both may be located close to the carotid bifurcation. The parathyroid IIIs may also descend along with the inferior thyroid veins, anterior to the trachea, and follow this pathway into the superior mediastinum. If they are located *outside* the thyroid sheath, they may occasionally pass infero-medially *behind* the oesophagus into the posterior mediastinum.[5–7] The usual relationship with the recurrent laryngeal nerves is as follows: The superior parathyroid glands are usually *dorsal*, while the inferior parathyroid glands are usually *ventral* to the recurrent laryngeal nerves.[8,9] When parathyroid tissue is located in very aberrant locations, it is unclear whether this represents the presence of supernumerary glands, entire glands that failed to migrate caudally, or these may even be ectopic glands that were formed from pharyngeal endoderm.

In embryos at about the 20 mm CR length stage, the parathyroid glands usually completely separate from the thymic rudiments, which then continue to descend to their normal definitive location. The third pouch therefore becomes separated into two distinct components: (1) a ventral derivative destined to become part of the thymus gland; and (2) the dorsal part destined to become its parathyroid derivative. After this stage, the two thymic primordia derived from the ventral parts of the left and right third pharyngeal pouches descend farther and usually fuse together across the ventral midline. In mice, the two thymic rudiments usually remain as two distinct entities and fail to fuse across the ventral midline. Shortly afterward, the definitive thymus descends into the superior mediastinum to become both a superior and a partly dorsal relation of the pericardium. With the subsequent descent of the heart and its associated pericardium, the thymus gland now tends to lie more ventral to the pericardium than previously. It has also been noted that the two halves of the definitive thymus gland, although now fused across the ventral midline, may be asymmetrical. Part may be attached to the upper pole of the thyroid gland of the same side by a cord of tissue, and very occasionally such a cord may extend rostrally to an even greater extent. At the site where the future parathyroid tissue separates from the thymic rudiment of the same side, a variable number of small tubules may occasionally be found. If present, these are termed *canals of Kürsteiner* and tend to be more closely related to the thymic than the parathyroid tissue. They are sometimes regarded as derivatives of the ultimobranchial bodies and thus as derivatives of the ventral part of the fourth rather than the third pharyngeal pouch. While initially described by Kürsteiner, they were subsequently classified by Gilmour.[10] For further observations on the early development of the various derivatives of the third and fourth pharyngeal pouches in the human, see Weller and Boyd.[3,11]

ORIGIN OF STRUCTURES DERIVED FROM THE FOURTH PHARYNGEAL POUCHES IN THE HUMAN

The fourth pharyngeal pouch eventually separates from the pharyngeal wall in a similar way to that described previously for the third pouch—by the rupture of the *ductus pharyngo-branchialis IV*. Similarly, the dorsal portion of the fourth pharyngeal pouch shows early signs of differentiation, at about the 8 mm CR length stage, to form the primordium of the parathyroid IV gland. The position of the parathyroid IV glands tends to be reasonably constant, as they are usually located midway along the postero-medial border of the thyroid gland and are usually either largely or completely surrounded by thyroid tissue, although they may occasionally be more rostrally located. The fate of the ventral part of the fourth pouch has been hotly debated over the years. While little doubt exists that it has a considerably greater volume than the cranial derivative of the fourth pharyngeal pouch, a number of suggestions have been made as to its eventual fate.

The situation is complicated by the suggestion of some authorities that at an early stage of development, continuity might exist between the fourth and fifth pharyngeal pouches. It is now generally accepted that the fifth pharyngeal pouch is at most only a transient structure in the higher vertebrates. If, however, it is assumed that the fifth pouch is invariably amalgamated with the fourth pouch, then it is appropriate that this should be referred to as the *caudal pharyngeal complex,* as has been suggested by some. Little doubt now exists that the parathyroid IV in the human is derived from the *dorsal* portion of this pouch. The fate of the ventral part of this pouch, however, is less understood. An inconstant component of the *ventral* pouch is believed to be the equivalent part of the third pouch that gives rise to the thymic rudiment. It is now believed that another component gives rise to the *ultimobranchial body*, although the view is commonly held that this may be derived from the fifth pharyngeal pouch. From whatever its origin, the ultimobranchial "body" initially becomes closely associated with the lateral part of the lobe of the thyroid gland and then becomes completely incorporated into it.[3,11,12] It is also now generally believed that when this occurs, the cells of the ultimobranchial body give rise to the C-cells (or parafollicular cells) of the thyroid gland. These cells are involved in the production of calcitonin (or thyrocalcitonin) in mammals,[13] and it

is now accepted that they also give rise to the calcitonin present in vertebrates other than in mammals.[14,15] Ultrastructural evidence confirms that the parafollicular cells observed in the thyroid of the embryonic sheep originate from the ultimobranchial bodies.[16]

The definitive position of the parathyroid IV is usually fairly constant. This is believed to be because the nonparathyroid part of the fourth pouch fuses with the thyroid gland, which tends to prevent its caudal migration into the superior mediastinum. Some variability does in fact exist with regard to its final position, however, but this may be species dependent. The gland is sometimes attached to the posterior thyroid capsule or occasionally may be embedded in the thyroid gland. It may be located close to the tracheo-oesophageal groove or even posterior to the oesophagus. The inferior thyroid artery usually supplies the gland, but more rarely the superior thyroid artery supplies it.

Some variability also exists in relation to the fate of the ventral part of the fourth pouch. Hamilton and Mossman,[1] for example, indicate the following:

1. It may completely disappear.
2. It may be transformed into thyroid tissue, and for this reason this part of the thyroid was formerly termed the *lateral thyroid*—the term used by Norris with reference to the ultimobranchial body that became amalgamated into the thyroid gland and dispersed within it to give rise to the parafollicular cells.[12]
3. It might occasionally be transformed into thymic tissue, and for this reason this part of the thymus is sometimes termed the *thymus IV.*
4. It may differentiate into cysts or tubular tissue, comparable to the canals of Kürsteiner of third pouch origin.
5. Most likely, it becomes amalgamated into the thyroid gland as the parafollicular or C-cells that are then responsible for the production of the hormone thyrocalcitonin associated with the regulation of calcium homeostasis.[17]

While all of the human embryonic material studied by Norris[12] possessed at least four parathyroid glands, others have observed as few as two glands and as many as eight in their pre- and post-natal material.[18] For observations on the normal variations in the position of the human parathyroid gland, see Millzner.[19] His paper was of critical importance when it was written. It pointed out that surgeons were surprised during the first few decades of the 19th century when a considerable proportion of postoperative patients who had previously undergone a thyroidectomy showed features of tetany. This was because the surgeons were unaware of the significance of the inadvertent removal of the parathyroid glands during this procedure. When the risk was explained to them, substantial amounts of the lateral and lateral portions of the anterior thyroid capsule were retained, and in many cases this avoided postoperative tetany. This paper drew attention to the very considerable variability in the location of the parathyroids, a proportion of which had been displaced anteriorly by the goitrous enlargement of the gland. Following awareness of the problems likely to be encountered by inadvertent removal of the parathyroids, the incidence of this occurrence dropped from at least 30% to nearer 3% during the early 1930s. The aberrant position of the parathyroids tended to involve the parathyroid IIIs to a greater incidence than the parathyroid IVs in a ratio of about 2:1.

ORIGIN OF THE SINGLE PAIR OF PARATHYROID GLANDS PRESENT IN THE MOUSE

Figures 5.1 through 5.4, 4.1, 4.2, 4.5, 4.6, and the accompanying DVD containing virtual slides illustrate the development of the mouse parathyroid gland. Because of the small size of the parathyroid glands in the mouse, their presence in close association with the thyroid glands is frequently

missed. "Due to the small size of the parathyroid gland in the mouse, it is sometimes absent in routine sections of the mouse thyroid in toxicity and carcinogenicity studies."[20] Despite this, they are invariably present and in the mouse are believed to be exclusively the derivatives of the dorsal component of the third pharyngeal pouch[21]; therefore, if the human nomenclature is followed, they should be termed the parathyroid III glands. In the mouse, because of species differences compared with the situation observed in the human, no parathyroid IVs have so far been observed. This is not, however, to state that they may never be observed in certain strains or in mutant varieties of mice.

As in the human, the derivative of the ventral part of the third pharyngeal pouch gives rise to thymic tissue. As indicated previously, in the human (and in the mouse) the two thymic primordia descend into the superior mediastinum, but in the mouse, by contrast to the situation observed in the human, the thymic primordia almost invariably remain as two distinct entities. While they are closely apposed across the ventral midline, no evidence has been observed during the prenatal period that they fuse together (Kaufman, unpublished observations). It is also relevant to note that all of the numerous immunohistochemical studies that have been carried out with mice indicate that the thyroid gland in this species contains parafollicular or C-cells that produce the hormone thyrocalcitonin. This strongly suggests that while the fourth pharyngeal pouch possesses a ventral component that gives rise to an ultimobranchial body, or an equivalent structure, its dorsal part does not appear to differentiate, under normal circumstances, into parathyroid glands, as undoubtedly occurs in the human. Whether a transient fifth pharyngeal pouch develops in this species has yet to be determined.

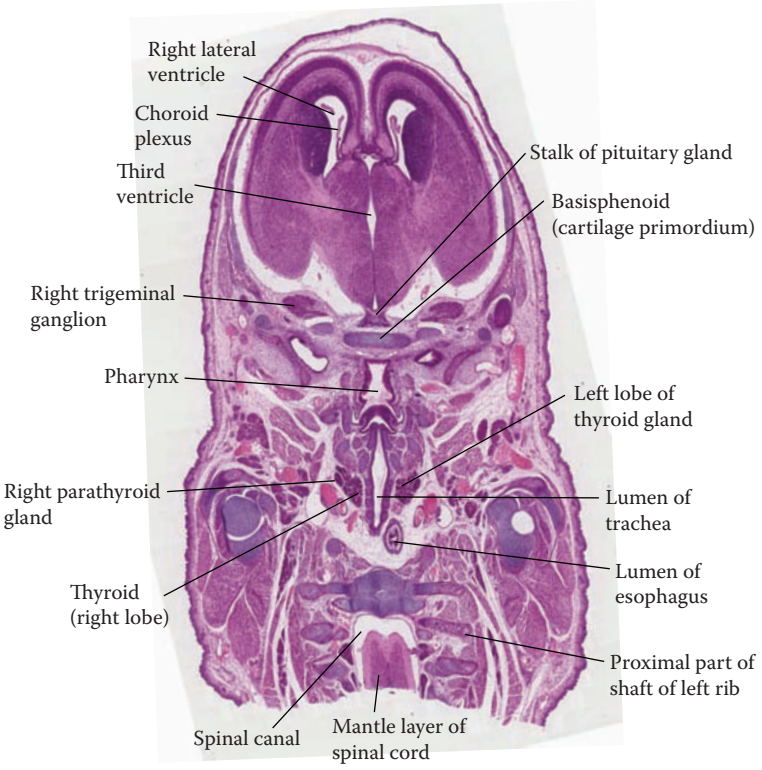

FIGURE 5.1 (E16.5_Fig5.1.svs) Low magnification, coronal section through the neck region of a mouse embryo (E16.5) stained with hematoxylin and eosin (H&E). The posterior part of the pharynx and lumen of the rostral part of the trachea are clearly recognizable, as are the right and left lobes of the thyroid and parathyroid glands. The right and left trigeminal ganglia are also clearly seen in this section.

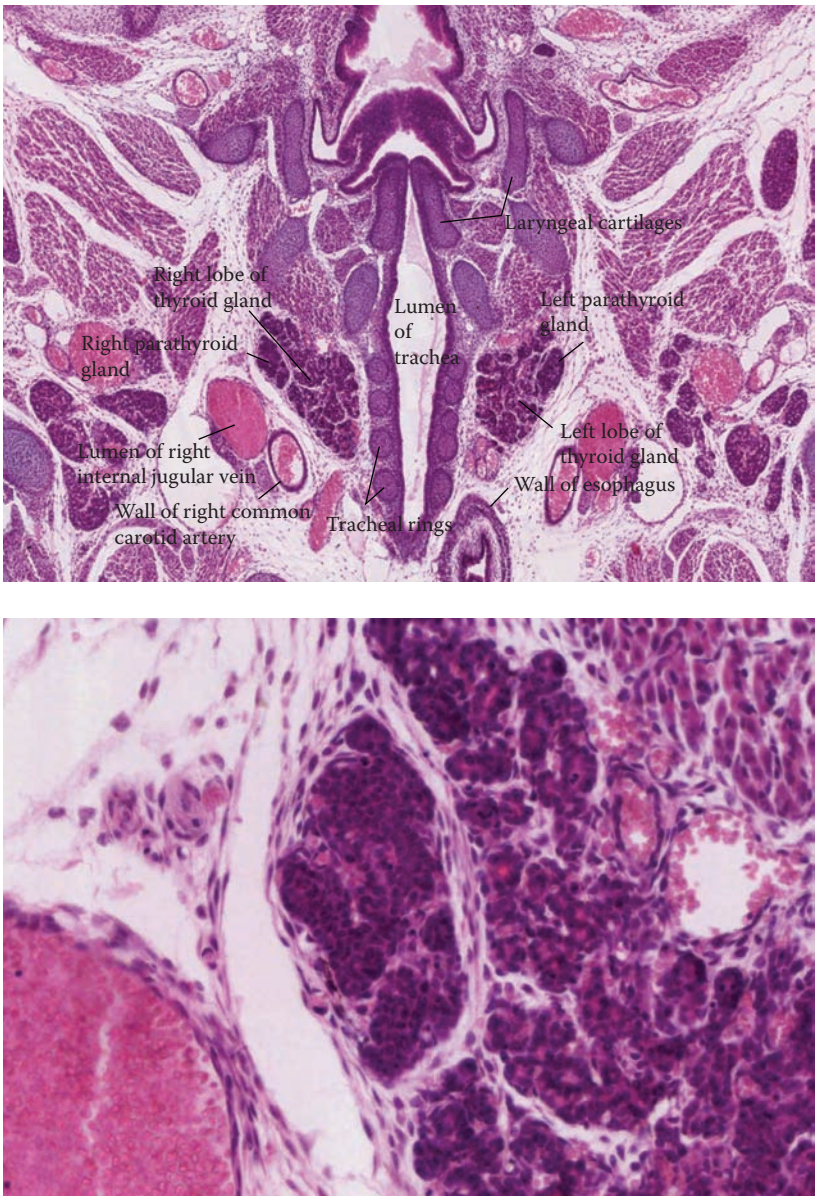

FIGURE 5.2 Medium (top) and high (bottom) magnification of Figure 5.1 shows both the left and right thyroid and parathyroid glands. Also note the close association between the parathyroid glands and the internal jugular veins, and the latter's association with the common carotid arteries in the medium magnification image. The close association between the medial surface of the thyroid glands and the tracheal rings are also clearly seen.

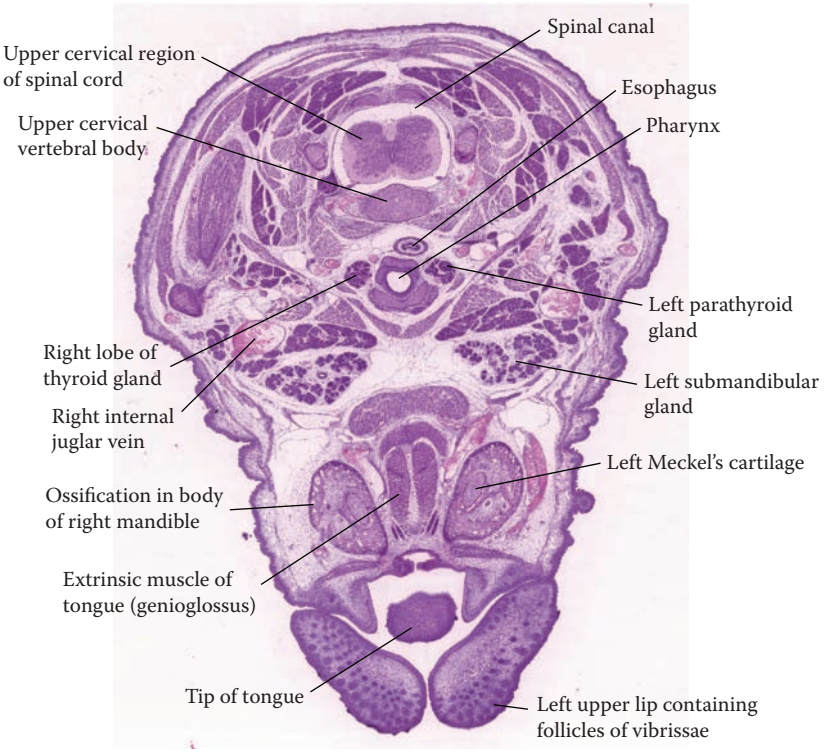

FIGURE 5.3 (E17_Fig5.3.svs) Low magnification, transverse section through the caudal part of the pharynx in the neck region and the two lobes of the thyroid gland of a mouse embryo (E17) stained with H&E. The location of the left parathyroid gland is also clearly seen.

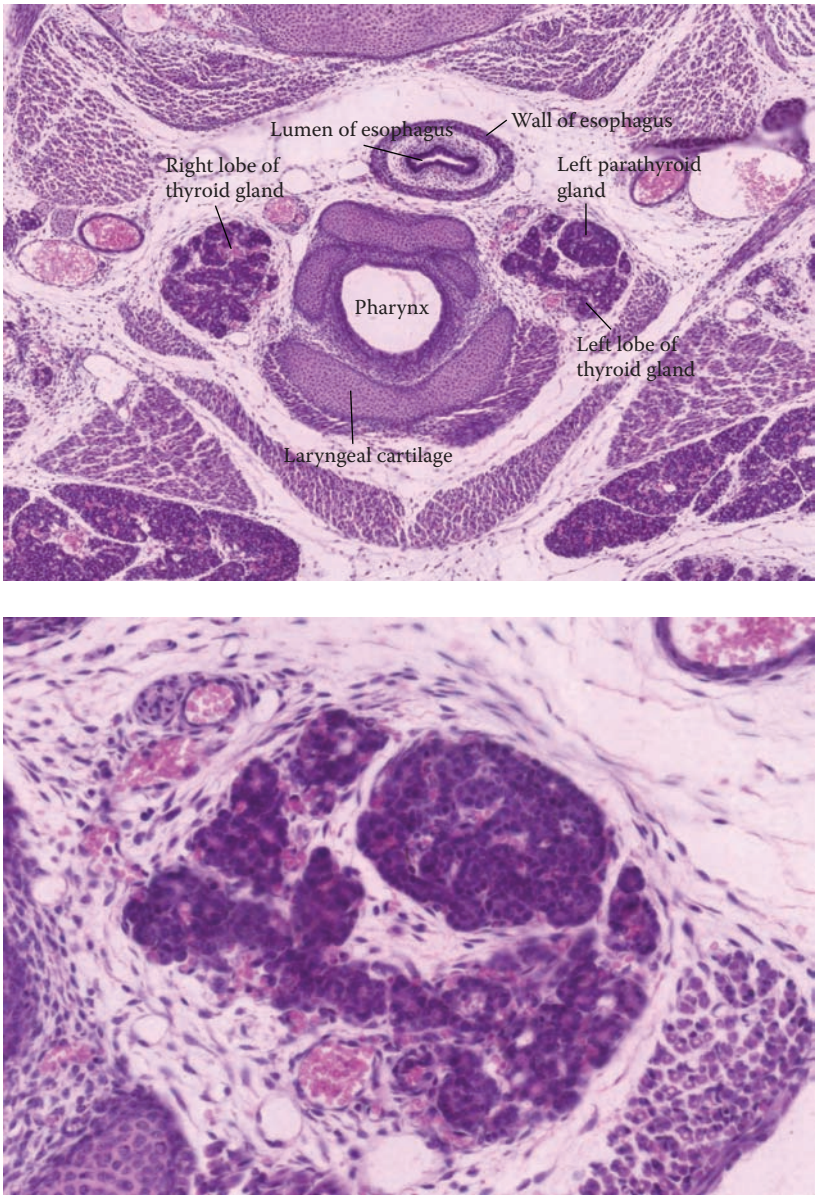

FIGURE 5.4 Medium magnification (top) of Figure 5.3 reveals the pharyngeal region showing its close proximity to the laryngeal cartilage. The left and right lobes of the thyroid gland are clearly seen, as is the lobe of the left parathyroid gland (see also high magnification, bottom). The close relationship between the lumen of the pharynx and that of the esophagus is also clearly seen.

An analysis of the literature on the number of parathyroid glands present in mice and in other rodents is both informative and somewhat confusing. According to the "Anatomy" chapter in the *Biology of the Laboratory Mouse*, the section that deals with the parathyroid gland[22] indicates the following:

> The position as well as the number of parathyroid lobes is variable, although usually a single lobe lies just under the capsule near the dorsolateral border of each lobe of the thyroid. Two members of a pair are seldom at the same anteroposterior level; sometimes one or both may be posterior to the thyroid[23] they may be deeply embedded in the thyroid tissue; and there may be more than 2.

This information is consistent with that in Roth and Schiller,[18] in which it is stated that the adult mouse "possesses a single pair of encapsulated, round-to-oval parathyroid glands,[24,25] which are found more superficially than those of the rat on the lateroventral or caudolateral aspect of the thyroid; though accessory glands have also been noted." For histochemical studies on some of the aberrant parathyroid glands associated with the thymus in the mouse, see Smith and Clifford.[26]

The analysis of histological sections through the cervical region of mouse embryos during the second half of gestation was informative only in that before TS 22/23 (about 14.5 dpc) it was not possible to distinguish the parathyroid rudiment from thyroid tissue with any degree of certainty. At and after this stage, the parathyroid gland appeared to be a quite distinctive entity and was often located either directly dorsal or even postero-lateral to the thyroid. Occasionally, the parathyroid gland was found in an aberrant location close to the common carotid artery.

PARATHYROID GLANDS PRESENT IN OTHER RODENTS

The *rat* appears to be similar to the mouse, in that it normally possesses a single pair of parathyroid glands (parathyroid IIIs). These are usually located on the dorsolateral border of the cranial pole of the thyroid gland and are either partially or completely embedded within it. However, embryonic nests were found in each thymic lobe in 50% of all of the adults studied by Van Dyke,[27] while others have reported that as many as 3–11 accessory glands may be located between the apex of the thymus and the chief parathyroid gland,[28] many being accompanied by epithelial-like cysts. Roth and Schiller[18] noted that numerous studies had been carried out on the development and ultrastructural features of the parathyroid glands in the rat. They also noted that secretory activity had been reported in both embryonic and fetal stages in this species.

The *guinea pig* generally has four parathyroid glands, although Pischinger[18,29] found only a single pair. Others have noted that the parathyroid IV is generally closely associated with the thyroid. A parathyroid V has also been noted in the guinea pig, while Jaffe[28] stated that there might occasionally be thymic tissue inside the parathyroid III in this species. Various numbers of parathyroid glands have been noted by a number of other authors.[18,28,30] By contrast, the *hamster* is said to possess two parathyroid glands,[18,31] and these are said to resemble those present in the rat.

ROLE OF NEURAL CREST IN PARATHYROID DEVELOPMENT

A number of indirect analyses indicated that neural crest mesenchymal tissue was probably involved in the formation of the vertebrate parathyroid gland. This topic has been extensively investigated using the methodology of Le Douarin.[32] She established, using chick-quail chimeras, that this is indeed the case. She demonstrated by the transplantation of quail prosencephalon, mesencephalon, and rhombencephalon into the chick host that neural crest tissue is involved in the development of the parathyroid, thymus, and thyroid glands and the ultimobranchial bodies. The quail cells formed all of the connective tissue of the thyroid and parathyroid glands except for the endothelium of their blood vessels.[33] It is now generally accepted that these avian findings such as those described by Le Douarin[32] and Noden[34] are also applicable to the events that occur during mammalian

embryogenesis.[35,36] However, minimal *direct* information has been obtained regarding the origin of the components of the parathyroid gland in the mouse until very recently, with the availability of molecular markers (see below). Assuming that the events that occur in the avian studies also occur in mammals, then the connective tissue present in the mammalian parathyroid glands is also derived from mesenchymal tissue of neural crest origin. Recent transplantation studies involving the rat,[37] although promising with regard to displaying evidence of cranial neural crest cell migration in this species, might one day be able to provide direct evidence of neural crest involvement in the formation of the parathyroid gland. Migration of labeled cells occasionally occurred into pharyngeal arches three and four, although significant differences were observed between the pathways of migration in the rat material and in the avian studies.[38,39]

Using the chick-quail methodology it was also possible to demonstrate that the branchial arches were colonized by neural crest-derived quail cells at stages before, during, and after the formation of the ultimobranchial body rudiment.[32,33] Studies of developmentally more advanced chimeric embryos revealed that the ultimobranchial bodies were formed by a mixture of cells from these two sources, but particularly from the neuroectodermal cells of quail origin. In a proportion of cases, the invading quail cells differentiated into glandular cells that possessed all of the cytoplasmic characteristics of C-cells, although the cells that lined the glandular cords were exclusively of host origin and only a proportion of the connective tissue elements were of quail origin. In some cases, only a few, or occasionally none, of the secretory cells in the ultimobranchial bodies were of quail origin. In the cases where the C-cells were largely of quail origin, it was possible to detect their origin through the use of appropriate immunocytochemical markers for calcitonin. This unequivocally confirmed that these C-cells were derived from the rhombencephalic neural primordium.[40]

What has yet to be established from these studies is whether the neural crest cells are preconditioned to a particular fate or whether the environment in which they find themselves following their migration is what stimulates them to differentiate in a particular direction.[32]

OBSERVATIONS ON THE MOLECULAR GENETIC CONTROL OF MAMMALIAN PARATHYROID DEVELOPMENT

Recent molecular genetic studies in both the human and particularly in the mouse have revealed that the development of the mammalian parathyroid as well as the thymus and thyroid glands are under genetic control. These studies, particularly those involving the analysis of mouse models for hypoparathyroidism and the control of pharyngeal development, have helped identify some of the critical genes that may be involved in parathyroid development.[41] For example, it has been noted that an insufficiency of one of these genes (termed GATA3) may be involved in human hypoparathyroidism, deafness and renal dysfunction, a condition termed the HDR syndrome. Furthermore, these studies indicate that this condition may be inherited as an autosomal dominant syndrome. GATA3 belongs to a family of zinc-finger transcriptional factors that are involved in vertebrate embryonic development, and this syndrome is consistent with the expression pattern of this gene during human and mouse embryogenesis, particularly in the development of the kidney, otic vesicle, and parathyroids.

Other genes, such as *Hoxa3*, also appear to play an important role in this pathway, as homozygous targeted mutant mice lacking this gene display a range of congenital defects, including an absent thymus and parathyroids as well as a reduction in thyroid size. Further studies have confirmed that the gene *Hoxa3* plays a critical role in the development of the parathyroid glands in the mouse.[42]

Homozygous mutant mice with deletions for *Gcm2 (Gcm2*^{tm1Kry}*)* also lack parathyroids but have a normal level of parathyroid hormone (or parathormone [PTH]) concentration. It was possible to demonstrate that PTH expressing cells located within the thymus produced this hormone. A deletion of this gene in a patient led to hypoparathyroidism.

In a similar study,[43] it was noted that mutations of the *EYA1* gene in the human patient caused the condition branchio-oto-renal (BOR) syndrome, while inactivation of the equivalent gene in the

mouse (*Eya1*^{tmRilm}) impaired the early development of a number of organs including the ear, kidney, and skeletal system. This gene in the mouse is expressed in all of the cells involved in the development of the thymus and parathyroid gland. This gene is also expressed in the fourth pouches, and in the presence of this gene mutation, which results from the absence of the product of the normal gene, failure of fusion of the ultimobranchial bodies with the thyroid gland occurs. Thus, thyroid hypoplasia is observed with a severe reduction in the number of parafollicular cells. Other genes were also shown to play a critical role in the differentiation of structures derived from the pharyngeal pouches.

HISTOLOGICAL AND ULTRASTRUCTURAL FEATURES OF PARATHYROID GLANDS

Because so little has been described of the histological and ultrastructural features of the parathyroid glands in the developing mouse, information available from comparable studies on the human gland is described first. This is followed by material on the cellular features of the parathyroid gland in the developing rat and then what is available on the prenatal mouse. These accounts are complemented by information on the comparable features noted in the adults of these species.

CELLULAR MORPHOLOGY OF THE PRENATAL PARATHYROID GLAND IN THE HUMAN

The first evidence of a parathyroid primordium is seen in the human embryo at the 8–9 mm CR length stage, when cellular proliferation occurs within the lumen of the dorsal component of the third pharyngeal pouch, although there appears to be no obvious thickening within the wall itself. The cells that are observed at this stage are large, having a diameter of about 7–12 μm. They possess a round or oval nucleus and are surrounded by clear cytoplasm. Shortly afterward, the first evidence of the parathyroid IV is seen in the dorsal part of the fourth pharyngeal pouch. The cells are identical in appearance to those destined to form the parathyroid III. At this stage, they are said to be similar in size to those of the developing thyroid gland. By the time that separation of the parathyroids from the pharyngeal wall has occurred, the overall shape of these glands is globular; however, as growth proceeds the glands' longitudinal axes enlarge, and they tend to become flattened.

At the gross histological level, the glands are now principally composed of reticular cells associated with connective tissue elements that separate the reticular cells into nests and cords. By the time the embryo has a CR length of about 100–150 mm (at about 20 weeks of gestation) vascular sinusoids have invaded the gland, and the epithelial cells are now seen to be more obviously separated into cords. A period of active epithelial cell growth, associated with an increase in number of cells, then occurs, and by the time the human fetus reaches about 230 mm CR length (at about 28 weeks of gestation) it is first possible to recognize specific cell types within the gland. The gland is now seen to contain darkly staining "chief" cells, while there is also an increase in the ingrowth of connective tissue and vascular elements. By the time of birth, a few fat cells are also recognized within the gland. Neither oxyphil cells nor definitive follicles are, however, seen during the prenatal period.

During the latter part of the fetal period, the parathyroid glands increase in volume, and this increase in their growth continues for the first three or four decades. At the end of this time, their growth ceases, and their overall weight in the adult human male is usually about 120±4 mg, while in the adult human female it is about 140±5 mg.[44] The glands measure about 6 × 4 × 2 mm each and vary in color from a dark tan to a yellow-gray tan.

In a recent study on human parathyroid gland development during the prenatal period, it was noted that from about midgestation onward there was an increase not only in the vascularity of the gland but also in the number of intercellular lymph spaces present.[45] The latter were observed both

within the parenchyma of the gland and in its capsule. Pezerovic-Panijan et al. suggested that the lymphatic system probably also plays a part in the transfer of parathyroid hormone into the circulation from the second half of pregnancy onward.

ULTRASTRUCTURAL MORPHOLOGY OF THE PARATHYROID GLAND IN THE HUMAN

The active secretory cells within the gland are the *chief* (or *principal*) *cells*, and these synthesize the PTH. The hormone is then transferred to the Golgi region within these cells and it is here that the hormone is packaged into the secretory granules.[18] From a detailed ultrastructural analysis of these cells, it appears that each chief cell undergoes cyclic changes independently of the adjacently located chief cells. In the pig, for example, it has been suggested that the secretory granules may be extruded from the chief cells into the perivascular space, although such a mechanism has not been noted in the human gland. Adenyl cyclase and cyclic 3',5'-adenosine monophosphate are believed to play an important role in the secretory process. These studies strongly suggest that adenyl cyclase is the mediator of the known effects of calcium ion on PTH.[46] Membrane-limited granules have also been observed in the extracellular space in the human and in the pig gland and have also been observed in the capillary endothelium of the human parathyroid gland.[18] While such granules have been observed in the chief cells of all species studied, there is no *direct* histochemical evidence that these granules contain parathyroid hormone. In the bovine chief cells, the larger bodies that contain acid phosphatase probably do not contain hormone. In the mouse, antiparathyroid antibody has been used to demonstrate the presence of hormone, and this appears to be uniformly distributed in the cytoplasm of the chief cells in many species, although no specific evidence of localisation was apparent. One of the principal problems encountered by many researchers up to the mid 1970s was that it was not possible to localize the hormone within any specific component of these cells. It was believed that this was because their antiserum was prepared against an impure preparation of parathyroid hormone. However, when a more specific antiserum was employed, Perkin et al. demonstrated that it contained antibodies to PTH. When this antiserum was employed in their study, they showed that the presence of hormone appeared to be localized in the intercellular spaces within the gland. Furthermore, specific immunofluorescence was not seen within parenchymal cells of the parathyroid gland.[47]

Most authorities consider that *oxyphil* (or *eosinophil*) *cells* are first recognized in the human parathyroid gland only from the time of puberty, although some have suggested that such cells were already present by the first 6 months after birth.[48] It is relevant to note here that these cells have been recognized only in the human, the rhesus macaque (*Macaca mulatta*), and cattle (*Bos taurus*). In the human, such cells either may be found in isolation dispersed among the chief cells or are present in small groups, where they are present in the form of nodules or follicles. These cells are about 6–10 μm in diameter and are readily recognized because of their small, dark, and centrally located nucleus. The latter is smaller than that of the chief cell, while its cytoplasm is eosinophilic, lightly staining, and granular. These cells increase in number with the age of the individual, and it has been noted that their cytoplasm is particularly rich in a wide range of enzymes. Another obvious feature of these cells is that their cytoplasm is filled with tightly packed and morphologically abnormal mitochondria, and this is said to give these cells their granular appearance on light microscopy. As endoplasmic reticulum and atrophic Golgi apparatus are only rarely encountered in their cytoplasm, it is believed that the oxyphil cells are not involved in hormone synthesis and secretion. However, the presence within them of large numbers of mitochondria strongly suggests that they have a very high level of metabolic activity.[49]

The vascular supply to the glands is principally through a series of profusely innervated medium sized vessels, mostly from the inferior thyroid arteries or anastomoses between the inferior and superior thyroid arteries. The nerve supply appears to be via sympathetic nerve fibers that travel

along the vessels and often terminate on the surface of the secretory cells. It is believed that the sympathetic supply is either from the superior or middle cervical ganglia or passes via a local plexus associated with the thyroid gland. The nerve supply is believed to be exclusively vasomotor and not directly involved in the control of hormone secretion, as this is controlled by variations in the calcium levels in the blood that passes through the glands (see below). The glands also possess a substantial lymphatic drainage, and this is closely associated with the lymphatic drainage of the thyroid and thymus glands.

Unlike the morphological appearance in the normally functioning parathyroid gland, patients with primary hyperparathyroidism with adenomas and chief cell hyperplasia, the oxyphil cells may contain abundant amounts of endoplasmic reticulum, large Golgi apparatuses and secretory granules. This appearance strongly indicates that these cells are capable of hormone synthesis and secretion. Such tumors and chief cell hyperplasia composed almost entirely of oxyphil cells are, however, only rarely encountered.[18,50,51]

ULTRASTRUCTURAL MORPHOLOGY OF THE RAT PARATHYROID GLAND

The first evidence of parathyroid material is observed in the rat on about 13 dpc, and the gland is shortly afterward invaded by vascular elements. While the parathyroid III is initially attached to the thymic rudiment, it tends to separate from it as soon as it comes in contact with the thyroid gland. As noted previously, a parathyroid IV is not noted in this species, although embryonic rests of parathyroid tissue are commonly observed in the adult rat within the thymus gland. During the prenatal period the parathyroid glands are composed of a single cell type. These so-called chief cells are round and measure about 6 μm in diameter. They soon become surrounded by vascular and connective tissue elements. Rosof[52] noted that these cells possess secretory granules that were more numerous close to the capillaries and that they become larger with advancing age. Such cells could be divided into two subtypes according to their staining characteristics following osmic acid fixation of glandular tissue. These were referred to as either *light* or *darkly staining* cells. The former generally constituted up to about 95% of the cell population in the gland and was believed to represent the resting phase of the cell's cycle. The darkly staining cells, by contrast, were believed to represent the secretory phase of the cell cycle and contained large numbers of filamentous mitochondria. This observation was later confirmed, and the granules present believed to represent the secretory product.[53]

Numerous ultrastructural studies have been carried out to investigate the secretory process in the rat and have generally confirmed the earlier finding of what appears to be the two chief cell subtypes, representing those at the synthetic phase and those at the actively secretory phase of the cell cycle.[54,55] In general terms, this is similar to the situation observed in the human parathyroid gland, although the presence of endoplasmic reticulum was only rarely seen in these cells in the rat compared to the situation observed in the various other species that have been studied. Secretory activity was observed in both embryonic and fetal rat glandular tissue.[18,56] It was also noted that the concentration of these two cell subtypes present in the glands maintained in organ culture appeared to reflect the ambient calcium concentration.

The situation in marsupials is unpredictable when compared to the situation in the human and in rodents, and the arrangement seen is often variable between even closely related species. Two pairs of parathyroid glands are usually present, one pair derived from the third pouch and a second pair from the fourth pouch. The size of the parathyroid III glands is also usually larger than the parathyroid IV glands. Curiously, while the parathyroid III gland is usually located close to the carotid bifurcation, the shape of this gland and its eventual location tends to be extremely variable. Unlike the situation in the human, it is the parathyroid IV glands that usually descend into the superior mediastinum and are associated with the thymus gland there, although in some marsupials while the parathyroid IV is seen in the pouch young it is absent in the adult. Furthermore, in none of the marsupials so far studied were the parathyroid glands at any stage during either the pre- or postnatal period closely associated with the thyroid gland.[18]

ULTRASTRUCTURAL MORPHOLOGY OF THE MOUSE PARATHYROID GLAND

As in the adult rat, the presence of a small or large Golgi apparatus in the parathyroid chief cells of the mouse reflect the stage in the secretory cycle, being associated with the resting (i.e. synthetic) or secretory phase, respectively. Similarly, chief cells of only a single type are recognized, although the amount of vascular and connective tissue present in the gland is considerably less than that normally present in the rat. The nucleus of the chief cells tends to occupy a larger proportion of the cell, and as a consequence, the volume of cytoplasm present is relatively small. At the ultrastructural level, numerous granules are present within the cell, and these tend to be smaller than those seen in the rat, although the number of secretory granules per chief cell tends to be extremely variable. It has also been noted that the granules present appear to originate in the Golgi apparatus, and then migrate to the margins of the cell. Their membranes then appear to fuse with those of the cell membrane, and the secretory material then passes into the extracellular space, and thence into the circulation. A similar secretory cycle has been observed in the mouse compared to that noted in other mammalian species.[18,57]

FUNCTION OF THE PARATHYROID GLANDS

The principal role of the parathyroid glands is the synthesis and secretion of PTH or parathormone. The secretion of PTH principally leaves the gland via the dense plexus of sinusoidal capillaries, although it has been suggested that a proportion may pass into the circulation via the components of the lymphatic system that ramify throughout the gland. During the secretory component of the cell cycle of the chief cells, the granules of these cells release their contents into one or probably both of these systems. PTH is principally concerned with the control of the level of circulating calcium and phosphorus. Other hormones, or hormone-like substances, such as calcitonin and 1, 25-hydroxy-cholecalciferol are also involved in this activity. The latter is produced by the action of heparin and renal cells on vitamin D.[58] The secretion of PTH is controlled by the level of calcium ions in the blood that pass through the parathyroid glands. Copp and Talmage[59] have described the hormonal control of calcium metabolism in detail.

PTH acts on osteocytes and osteoclasts, principally by stimulating osteolysis. If a high level of secretion is maintained, this is believed to stimulate bone remodeling. This hormone also affects renal ion transport by increasing the excretion of phosphate, sodium and potassium, and decreasing the excretion of calcium. 1, 25-hydroxycholecalciferol is believed to regulate the action of PTH, although exactly how PTH affects its target cells is unclear.

In order to maintain appropriate levels of calcium in the plasma pool and in the extracellular fluid, gastrointestinal, renal, and skeletal systems are brought in and play a critical role. These activities are controlled by the hormones released by the parathyroid glands and by the C-cells of the thyroid gland. Any deviations from the appropriate level in the blood prompts changes in the controlling signals from these sources in an attempt to restore the level of plasma calcium to within a narrow range of about 1.01–1.26 millimole/liter. The control mechanism involves a negative feedback loop to help maintain control of this level. If a sudden reduction in the level of serum calcium occurs for any reason, an increased release of PTH occurs. This has the effect of stimulating renal tubular calcium reabsorption and skeletal calcium release. If the calcium level remains lower than the desired level for a period of hours or days, then an increase in hormone release will also stimulate an increase in gastrointestinal calcium absorption associated with osteoclast-mediated bone resorption. If, by contrast, the circulating level of calcium increases, the level of PTH release decreases, with the opposite effect. If this occurs in young animals, an immediate release of calcitonin occurs, to reduce further reabsorption from the skeleton.[60] Observations on the interaction between PTH and calcitonin are discussed in further detail in Chapter 4, Thyroid Gland.

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6 The Pancreas

INTRODUCTION

The pancreas has two quite distinct functions. Many, though not all, investigators believe that both components of this organ are derived from the same primitive cells. A substantial number of recent studies would appear to indicate that the pancreas contains endodermally derived pancreas-specific precursor cells and that both the islets and the parenchymal acinar tissues are in fact derived from a common precursor (see below).[1,2] While one of the roles of the pancreas is as an *exocrine* organ, and therefore part of the digestive system, its other role is that of an *endocrine* organ. In the human, its development and gross anatomy are relatively easily understood, principally because its various component parts are reasonably well delineated. In rodents, the gross anatomy of the pancreas is far less easily determined, as it consists of a series of irregular lobes and lobules that extend to occupy the available space between a number of the intra-abdominal viscera. More particularly, its component parts in rodents are quite difficult to define both during the pre- and early post-natal period and in the adult. The exocrine part of the gland produces a secretion with a relatively high pH (range 7.5–8.8). The low pH of the gastric contents (adult human males, average pH value 1.92; adult human females, average human pH value 2.59) tends to be neutralized by the alkalinity of the pancreatic and biliary secretions (hepatic bile pH range 6.2–8.5; gallbladder bile pH range 5.6–8.0).[3]

It has been suggested that the histological features and mode of development of the pancreas are similar in mammals, birds, reptiles, and amphibians, while in some fish the islet cells are segregated as Brockmann bodies. Invertebrates do not possess a pancreas, although comparable endocrine cells are found in the gut or the brain.[4] It has also been noted that the endocrine cells start to differentiate before the exocrine cells, and co-expression of different hormones by the same cell is often seen at early developmental stages. While endocrine cells produce certain gene products that are characteristic of neurons, evidence from chick-quail chimera studies indicates that they are endogenous and not of neural crest origin. Furthermore, it appears that there is a continuous slow turnover of cells fed from a stem cell population in the ducts so that the control of their production is likely to be local rather than systemic. Species differences are particularly marked in mammals in relation to the drainage of the exocrine pancreas (also see below).[5]

In 1973, Pearse et al.[6] proposed a hypothesis that neural crest-derived material was carried into the pancreatic anlagen and progressively replaced the pleomorphic granules already within the α type cells (also termed A cells or A_2 cells, which secrete *glucagon*), β type cells (also termed B cells, which secrete *insulin*), and δ type cells (also termed D cells or A_1 cells, which secrete *somatostatin*) by spherical granules of neuroectodermal origin. Therefore, a series of chick-quail studies were undertaken.[7] These were exclusively designed to investigate the fate of the neural crest cells that migrated into the host pancreas. Fontaine et al. observed that the quail cells in the chick host pancreas were always separate from the host exocrine and endocrine structures. The crest cells that migrated into the pancreas differentiated into parasympathetic ganglia rather than into islet structures. In subsequent studies,[8] antisera directed against glucagon, insulin, and somatostatin (marking α, β, and δ cells, respectively) were applied to the chimeric pancreata, and it was noted that the endocrine cells never displayed the graft marker. Identical findings were reported by Pictet et al.[9] when similar studies were carried out to investigate the possible neural crest origin of the islet cells in the rat embryo.

As noted above, various distinct cell types may be recognized in the islets. Although these usually require specific staining methodology, more modern techniques involve the use of appropriate antibodies. The various cell types present in the islets include the A or α_2 cells that secrete *glucagon*, the β or B cells that secrete *insulin*, and the δ or α_1 or D cells that secrete *somatostatin*. Other cell types have also been observed, including the F or PP cells that secrete *pancreatic polypeptide*.[10–12] All these cells tend to be restricted within the islets. The α cells, for example, tend to be located principally at the periphery of the islets, while the β cells are principally located in the center of the islets and make up about 70% of their mass. The F cells are relatively rarely encountered and, while principally scattered throughout the islets, may also occasionally be found among the acini.

It is relevant to note here that additional cell types have also been described in the pancreatic islets. For example, in 1937, Thomas[13] described in the islets of the opossum an "E" cell with secretory granules larger than α granules and with a specific trichrome staining reaction. Munger et al.[14] confirmed the presence of these E cells in the opossum islets prepared for electron microscopy. It appears[13] that the E cell has not been reported in other species. It has also been noted that the "C" cell of the pancreatic islet lacks secretory granules. These C cells had been observed[13] and were believed to be either a discrete cell type or a functional phase of an α or β cell. More recent studies[15] did not indicate the presence of these cells in guinea pig islets. Other studies provided convincing evidence that the C cell of the guinea pig was really the δ cell.[16] Other cell types have also been described[17,18] in the pancreatic islets. According to Lacy and Greider,[10] these have yet to be defined by electron microscopy and need to be confirmed by studies that relate these cells to a specific function.

Lacy and Greider[10] also noted that ultrastructural evidence had been cited as confirmation of an acinar-islet cell type occurring in the pancreas. According to these authors, the origin and possible function of these cells are a mystery, and that "*their rarity makes them unlikely candidates for study.*"

According to *Gray's Anatomy*,[19] peptide-secreting cells, with smaller granules than those in A, B, and D cells, occur in the human pancreas in at least two forms: One contains pancreatic polypeptide (PP cell), and another has an ultrastructure like that of D_1 cells of the gastric mucosa. Pancreatic D_1 cells differ from PP cells in the diameter of their granules: The mean granule diameter of D_1 cells is 118 nm, while that of PP cells is 141 nm. D_1 cells do not react with antibovine pancreatic polypeptide serum, while the granules of PP cells react with this serum.[20] Although the product of gastro-enteric and pancreatic D_1 cells is uncertain, it may be related to vaso-active intestinal polypeptide (VIP).[21] It should also be noted that both D_1 and PP cells are not restricted to pancreatic islets, being also scattered throughout the predominantly exocrine tissue.

The majority of the human's exocrine secretion enters the middle of the second part of the duodenum through the main pancreatic duct (of *Wirsung*). This enters the duodenum postero-medially at the so-called greater (or major) duodenal papilla, where it often enters the duodenum at a common opening with the common bile duct. The latter is often relatively short and termed the *hepatopancreatic ampulla* (or *ampulla of Vater*), and the opening and closing of the latter is controlled by the *sphincter of Oddi*. In a proportion of individuals the two ducts enter the duodenum separately. Relatively frequently, an "accessory" pancreatic duct (of *Santorini*) may also be present that also enters the second part of the duodenum at the so-called lesser (or minor) duodenal papilla. If present in humans, this is usually about 6 cm in length and located about 1–2 cm rostral and anteromedial to the site of entrance of the main pancreatic duct. This duct drains the lower part of the head and uncinate process of the pancreas and ascends in front of the main duct with which it may communicate before opening into the lesser duodenal papilla (discussed later herein).[22] The daily secretion produced by the exocrine pancreas is considerable and plays a critical role in the digestion of food. It is said to normally drain about 1,200 ml of an enzyme-rich fluid each day into the duodenum.[11,12]

In rodents, there tend to be substantial but often various numbers of pancreatic ducts, the majority of which drain into the common bile duct, usually at irregular intervals along its length. One or more of these pancreatic ducts enter the duodenum close to the site of entrance of the common bile duct. In both the human and in rodents, special circumscribed regions of the pancreas possess an endocrine function. Despite their considerable number, these localized regions of the pancreas,

called *islets* (or *islands*) *of Langerhans*, occupy a relatively small proportion of the gland. These contain a number of specialized cells that are involved in the secretion into the circulation of a number of hormones, including insulin and glucagon and a number of other hormones described in detail herein. These hormones are principally involved in the metabolism of carbohydrates.

DEVELOPMENT OF THE EXOCRINE PANCREAS IN THE HUMAN

Only a relatively small number of comprehensive studies have been undertaken that describe in any detail the development of the human pancreas. These draw attention to the means by which the definitive features of the adult gland are achieved, usually from the analysis of large numbers of human embryos and fetuses isolated at sequential stages throughout gestation. In one of the most detailed studies, Liu and Potter[23] analyzed histologically a total of 130 human embryos and fetuses ranging in age from 5 weeks of gestation to full term. The findings of O'Rahilly[24] in his brief review of the sequence of events observed with regard to the development of the pancreas during the embryonic period proper—from conception to about the middle of the eighth week of gestation—complement the information previously provided by Liu and Potter.

In all mammals, as in other vertebrates, the liver primordium is the first of the anlage to develop at the foregut-midgut junction (from the biliary or hepatic outgrowth or diverticulum).[25] According to O'Rahilly,[24] the first evidence of proliferation at the foregut-midgut junction in relation to the pancreatic primordia is seen in human embryos with a Crown-Rump (CR) length of about 3–5 mm. This is the dorsal pancreatic primordium and appears as an outpouching of the endodermal lining of the dorsal wall of the duodenum. Such embryos, at Carnegie Stage (CS) 12, possess about 21–29 pairs of somites and are at about 26 days *post coitum* (dpc).[26] By contrast, the first evidence of a ventral pancreatic rudiment has been observed in embryos at CS 13 with a CR length of about 4–6 mm. These possess 30 or more pairs of somites and are at about 28 dpc.[27] This tends to be located at a point slightly caudal to the dorsal outpouching. Further development has been reported in relation to the ventral pancreas in embryos at CS 14, by about 32 dpc. By this stage, the latter appears to be an evagination from the bile duct (the hepatic diverticulum),[28] or it may open directly into the duodenum. While usually described as being an unpaired structure, others have reported that it might be either bilobed[29] or possess multiple lobes at this stage.[30] In embryos of about 8–11 mm CR length, at CS 16, at about 37 dpc, both the dorsal and ventral rudiments are now said to be contiguous,[24] although others have reported that this is first seen in embryos of about 11–14 mm CR length, at CS 17, at about 41 dpc.[26] It has also been suggested that there might also be evidence at this stage of fusion between the dorsal and ventral ducts.[31]

In a similar study by Park et al.,[32] comparable findings were reported in relation to the early development of the dorsal and ventral pancreatic primordia. It was also noted that evidence of rotation of the ventral pancreas was first observed at about CS 15 and completed by CS 17. It was also noted that by CS 18 the connective tissue that surrounds the pancreas had differentiated.

As far as the fate of the dorsal and ventral pancreatic rudiments is concerned, Russu and Vaida[31] indicated that the body and tail of the pancreas were derived from the dorsal rudiment, while the dorsal part of its proximal region gives rise to the upper part of the head. By contrast, the lower half to two thirds of the head is formed from the ventral primordium. They also noted that the uncinate process formed only after the fusion of the dorsal and ventral primordia. This view was in general agreement with that provided by Liu and Potter.[23] However, it differed from an example described by Bossy,[33] where an uncinate process formed despite the fact that no evidence of fusion of the dorsal and ventral primordia had occurred. They also indicated that the dorsally derived portion of the pancreas grew more rapidly than the ventrally derived portion and extended into the left side of the dorsal mesogastrium. The proximal part of the main duct of the ventral pancreas then usually opened into the distal part of the common bile duct or occasionally directly into the duodenum in an approximately similar location.

With the rotation of the duodenum, both the common bile duct and the ventral pancreatic rudiment are carried around the right and posterior side of the duodenum. The ventral pancreas then

extends behind the duodenum and subsequently fuses with the caudal part of the dorsal pancreas. By this means, most of the future head region is formed, although, as indicated above, the dorsal pancreas forms only the upper part of the head of the pancreas.[34] As a result of these changes in position, the distal part of the main duct of the ventral pancreas usually communicates with the main duct of the dorsal pancreas some distance along its length to form the main pancreatic duct (of Wirsung). As a consequence, the proximal part of the dorsal duct where it enters the duodenum usually regresses but may remain as the accessory duct of Santorini; however, for variations in the pattern of duct formation, see below. All of these configurational changes occur by the time embryos have a CR length of about 15 mm. Similarly, by this time, there is no remaining evidence to indicate that the different parts of the definitive pancreas are of different origins. Over the next month or so, the main duct elongates, and the first evidence of numerous side branches is seen. This also represents the first evidence of the characteristic lobular arrangement of the pancreas.

While the luminal epithelium of the main duct is columnar, toward the peripheral regions of the smaller branches, the cells lining them become progressively more cuboidal. During the fourth month of fetal development, greater evidence of lobulation is seen. At this stage, the lobules tend to be small and ovoid or even triangular in shape and are separated by large amounts of connective tissue. During subsequent fetal development, the volume of the interlobar connective tissue decreases while the lobules themselves become more compact. Later, they gradually coalesce to form larger lobes. This is particularly seen toward the time of birth, by which time only narrow strands of connective tissue separate the lobes. The islets of Langerhans also tend to be located toward the center of the lobes with the acini at their periphery. The latter now form the secretory elements of the pancreas and are first clearly evident during the third month of fetal development. Their cells have basophilic cytoplasm. While their apices converge toward each other, the most peripheral of the acini do not yet possess well-defined ducts, although their future location can be just discerned at this stage.

As duct proliferation occurs, new acini are constantly being formed and bulge from the walls of the future ducts that then develop a narrow slit-like lumen. The cells that line these ducts are then surrounded by acinous cells, and the former are then termed *centroacinous* cells. These possess a clear and colorless cytoplasm. The parts of the ducts that are proximal to the centroacinous cells increase in length and form the intercalated ducts. These then lead into the *intralobular* ducts that in turn empty into the *interlobular* ducts, which then empty into the main pancreatic duct. This is the basic arrangement seen in the exocrine component of the pancreas at full term.

GROSS ANATOMY OF THE PANCREAS IN THE ADULT HUMAN[19]

The human pancreas has a relatively soft consistency and is grayish-pink in color. It measures between about 12–15 to 20–25 cm in length and extends transversely across the upper part of the posterior abdominal wall. The gland usually weighs about 100–150 g. It is a retroperitoneal structure and extends on its right side from the concavity of the duodenum to the hilar region of the spleen on the left side. Its head region is directed to the right side, while its relatively narrow tail region extends to the left and ascends slightly as it crosses the posterior abdominal wall. It is located behind the stomach, being separated from it by the lumen of the so-called lesser sac. The neck region of the gland is situated at the level of the transpyloric plane, being located behind the pylorus (or outlet region) of the stomach. The gland possesses a number of poorly defined component parts—head, uncinate process, neck, body, and tail—all briefly defined in the following sections.

The Head and Uncinate Process

The head of the pancreas is bordered superiorly by the transversely running first part of the duodenum, while its other boundaries are, to the right, the medial part of the second or descending part of the duodenum. Its inferior boundary is the transversely running third part of the duodenum. The uncinate process runs from the lower and left part of the head of the pancreas and projects caudally

behind the superior mesenteric vessels. The vascular supply to the head and uncinate process is principally from the superior and inferior pancreaticoduodenal arteries. The former is derived from the common hepatic artery via the gastroduodenal artery, and this anastomoses with the posterior branch of the inferior pancreaticoduodenal artery. The latter comes either directly from the superior mesenteric artery or from its first jejunal branch. The superior mesenteric vessels cross the middle of the anterior surface of the uncinate process. The inferior vena cava is a posterior relation of the head and ascends behind it, covering a considerable proportion of its width. It is also related postero-superiorly to the right crus of the diaphragm and posteriorly by much of the right renal vein. Both the head and uncinate process of the gland are principally derived from the ventral pancreatic primordium, being drained by the duct of the latter.

The Neck

This part of the pancreas is where the head of the pancreas merges with the body and is relatively poorly defined. Its right boundary in the front of the gland (with the head of the gland) is the groove for the gastroduodenal artery. Its left boundary from behind (with the body of the gland) is the deep groove where the superior mesenteric and splenic veins unite to form the portal vein. Anteriorly, it is related to the region of the pylorus, while posteriorly it is related to the superior mesenteric vein and the first part (or caudal origin) of the portal vein.

The Body

This poorly defined region merges with both the neck and tail of the gland and has antero-superior, posterior, and antero-inferior surfaces. All except for the posterior surface are covered by peritoneum.

The Tail

This region is continuous with the body of the gland on its right side and narrows as it passes to the left toward the hilar region of the spleen. This is on its visceral surface where the branches of the splenic artery enter and the splenic vein emerges from this organ. The pancreatic impression, if present, is created by the tail of the pancreas and is located toward the lateral part of the hilar region and the colic impression. The tail of the pancreas lies within the lienorenal ligament and with the body is largely derived from the dorsal pancreatic primordium. The body and tail of the pancreas receive numerous arterial branches from the splenic artery. The venous drainage of the gland is into the portal vein principally via the splenic and superior mesenteric veins.

The Main Pancreatic Duct

This represents the exocrine drainage of the upper part of the head, body, and tail of the gland and is derived from the distal part of the main duct of the dorsal pancreatic rudiment. It is usually located nearer the posterior than the anterior surface of the gland. In the commonest arrangement observed, when it reaches the neck of the gland, it becomes directed downward and to the right toward the common bile duct, which lies on its right side. It then drains into the greater duodenal papilla, the former proximal part of the duct of the ventral pancreatic rudiment.

Hjorth[35] noted that in the male the accessory duct opened into the duodenum in 44% of the 50 cases he studied, while this only occurred in 14% of the 50 female pancreata studied. He suggested that occlusion of the main pancreatic duct was therefore more likely to lead to a blockage of the pancreatic outflow in the female than the male. It was suggested that this might be the explanation for the higher incidence of pancreatitis observed in the female.

To study this point in more detail, Dawson and Langman[22] analyzed the duct pattern by dissection supplemented by radiological techniques in pancreata isolated from 120 adults, 20 fetuses, and

20 infants. They showed that there was no significant difference between males and females in the incidence where the minor papilla was able to act as a safety valve in cases of occlusion of the major papilla. The proximal part of the original dorsal duct remained patent and entered the duodenum at the minor duodenal papilla, while the proximal part of the ventral duct joined the distal part of the original duct of the dorsal pancreas to form a "combined" pancreatic duct, the term suggested as an alternative for the "main" pancreatic duct now in standard use. The situation described here in about half of the cases examined. Dawson and Langman also observed cases where there was no communication between the ducts of the original dorsal and ventral pancreatic rudiments, called the *embryonic* arrangement. Similarly, they observed cases of an "ansa pancreatica," in which the inferior branch of the ventral and dorsal pancreatic ducts formed a loop entering the duodenum at the minor duodenal papilla, and documented other cases in which the accessory duct was either completely or partially obliterated.

HISTOLOGICAL STRUCTURE AND PHYSIOLOGICAL ROLE OF THE EXOCRINE PANCREAS[11,12]

With regard to the first evidence of secretory function of the parenchymal cells, evidence from the presence of fine secretory granules suggested that secretion was probably initiated during the fourth to fifth month of human fetal life and also that this was probably associated with the release of pancreatic fluid into the fetal intestine at this time.[36] Numerous studies have been published on the exocrine pancreas, but for a particularly comprehensive review of the literature in this field covering the period up to the end of the 1980s, the interested reader should refer to Schultz et al.[37]

Because of the very considerable variety of proteins produced by the exocrine pancreas, numerous ultrastructural and biochemical investigations have been carried out to determine the role of the various acinar cell organelles in their synthesis. The principal sites of synthesis within the acinar cells are the ribosomes, and these are closely associated with the membranes of their endoplasmic reticulum. The proteins released include both proenzymes and enzymes and are packaged in the form of small vesicles that bud off from the endoplasmic reticulum. These vesicles then fuse with components of the Golgi complex where some contribute to its protein constituents, while others are enclosed in vesicles that are condensed to form vacuoles. The latter form zymogen granules and are temporarily stored within the acinar cells. These cells secrete a wide variety of enzymes that are principally involved in the digestion of the ingested food.

To avoid a reaction with the cellular components of the gland, the pancreatic enzymes are secreted in an inactive form and are activated only once secreted into the lumen of the intestine. Trypsin, for example, is synthesized as the inactive trypsinogen, and this is activated only as and when required. Occasionally, in the condition of acute pancreatitis, autolysis of the cells of the pancreatic parenchyma may result when, for example, a gallstone blocks the main pancreatic duct. To avoid autolysis of the pancreas by trypsin, the acinar cells also produce a trypsin inhibitor, and the volume produced is usually adequate for this purpose.

The production of trypsin is produced periodically to coincide with its need during the digestive cycle. Another stimulus for the release of pancreatic enzymes is the presence of gastric contents in the duodenum. The release of two intestinal hormones, secretin and cholecystokinin, usually in combination, when transferred via the vascular system from the duodenum and jejunum to the pancreas also stimulates the secretion of a large volume of pancreatic fluid. This contains a high level of bicarbonate and greatly assists in neutralizing the activity of the gastric contents.[38–40]

COMPARATIVE OBSERVATIONS ON THE DEVELOPMENT OF THE ENDOCRINE PANCREAS

In 1903 Pearce was the first individual to study the pancreas of human fetuses. These fetuses measured from 2 to 21 cm in CR length.[41] Other studies on the development of this organ have been

carried out in a range of mammalian and nonmammalian species, including the sheep,[42] rabbit,[43] rat,[44] pig,[45] guinea pig,[46] and frog,[47] and attention is drawn to the information provided in some of these earlier studies at appropriate stages of this chapter. This is not to state that their findings are all still acceptable, only that they were some of the earliest studies undertaken in this field.

Somewhat surprisingly, a considerable body of literature is available on the islets of Langerhans. One of the earliest studies was published by Pearce in 1903[41] and involved the analysis of human embryonic and fetal pancreata ranging from 20 to 210 mm in CR length, with probable ages of between about 47 and just over 200 days of gestation. Other preparations examined in this study were pancreata from newborn individuals with congenital syphilis.[41] Other informative studies on the pancreatic islets have included those of Gomori,[48] Hard,[44] Ferner,[49] and Robb.[50]

Falkmer and Patent[51] published one of the most comprehensive overviews of the development of the pancreatic islets. While this is principally a comparative study, it draws attention to the fact that there was at that time a controversy based on the findings of Wessels[52] in the mouse embryo as to whether the islet cells were of mesodermal or endodermal origin. He had noted that the islet cells in the mouse became distinct only after the endodermally derived pancreatic primordia were invaded by mesodermal elements. This contrasts with the more traditional belief that the islets originate in the endodermally derived endothelial cells of the pancreatic ducts. To date, the critical aspect of their development has yet to be unequivocally established.

In an early study, β cells had been demonstrated histologically in the rat on the 17th to 18th day of gestation, in the hamster on the 11th day of gestation, and in the rabbit on the 30th day of gestation (just before or after birth).[53] In a subsequent paper, Grillo[54] used bioassays of extracts of the pancreata of fetuses from normal animals. These revealed that insulin was present in the pancreata of these fetuses of rodents before mature β-granulation could be detected microscopically. They further revealed that the presence of insulin or insulin-like substance was detected in the 14-day rat, the 18–19-day rabbit, the 16-day mouse, and the 11-day hamster fetuses.

It is relevant to note that calculations have been made to determine the number of islets present in the normal rat and human pancreas. According to Hellman,[55,56] the number of islets present in the newborn rat was about 243±21, and that this increased to about 3320±184 in older rats, at about 480 days of age. In most age groups studied, there was a statistical correlation between body weight and number of islets present in the pancreas. The total number of islet cells in the adult human has been calculated using a variety of methods. Clark, for example,[57] calculated that there were between 120,000 and 1,700,000 islets present, while Ogilvie,[58] using a different method, calculated that there were between 117,000 and 2,325,000 islets present. These findings strongly suggest that considerable individual variation exists in the total number of islets present between normal individuals.

ISLET FORMATION IN THE ENDOCRINE PANCREAS IN THE HUMAN

According to some authorities, the first islets are observed in the human embryo as early as at about 7–8 weeks postconception (pc),[23] while others did not recognize them before 9–10 weeks pc.[59] Most authorities, however, indicate that it is only by about the 10th to 11th week of human fetal development, in fetuses with a CR length of about 45–49 mm, that the islets of Langerhans are first readily recognized. The latter develop from small groups of cells with a darkly staining cytoplasm recognized among the clear epithelial cells of the ducts and appear to bud off from them. From the beginning, α and β cells can be recognized in these islets. Such budding from the cells that line the ducts occurs throughout the fetal period. The first evidence of insulin in the β cells can be detected as early as about 12 weeks of development[60,61] in fetuses in nondiabetic controls. Shortly afterward, granules may be recognized in both the α and β cells.[23,50,62] In the cytoplasm of the β cells, the secretory granules are usually concentrated in the region of the cell adjacent to the nearest blood capillary, while in the α cells the granule tend to be more evenly distributed throughout the cytoplasm.

In Conklin's study,[62] pancreata were isolated from fetuses ranging in size from 29 to 360 mm in CR length (corresponding to an age range of between the 8th and 39th week of gestation). The

preparations were fixed, and appropriately stained. This study indicated that the islet cells developed sequentially, with the argyrophilic cells appearing during the 8th week followed by the α, δ, and β cells during the 10th, 11th, and 13th weeks, respectively. The argyrophilic cells were considered to be intermediate in the formation of the α cells, while the δ cells were considered to be the precursors of the β cells. The α and β cells were considered to be "fixed" cell types, and transformation of one type to the other was not observed. From the end of the fourth month, the β cells exhibited morphological evidence of secretory activity, while the α cells displayed evidence of activity only after the early part of the fifth month. It was also suggested that the acini displayed no evidence of secretory activity during the fetal period.

In Grillo and Shima's study,[61] insulin was demonstrated by the fluorescent antibody technique and by immunoassay in the pancreatic islets of all fetuses of over 80 mm in CR length, while no insulin was present in extracts of the pancreas of 35 mm fetuses. It was also noted that insulin formation started before β granulation. It was also suggested that the development of the islets and the differentiation of their cells preceded the beginning of glycogen function in the liver of human fetuses. According to Steinke and Driscoll, fetuses isolated from diabetic mothers contained more insulin than those isolated from the control mothers.[60] It is relevant to note that Kim et al.[63] obtained a somewhat different outcome when the pancreata of fetuses isolated from alloxan-treated induced diabetic female rats were analyzed. A likely explanation for this different finding has, however, been suggested (see below).

In Falin's study,[64] the pancreata of a total of 20 embryos and fetuses were examined, ranging in CR length from 14 to 240 mm (approximately 5–30 weeks in age). In a table in this paper, the author cited many previously published researchers on this topic and noted that most believed that the age of fetuses in which the first islets were found was between about 10 and 12 weeks. Falin also noted that from the first evidence of islets, both α and β cells are seen and that they both display secretory granules in their cytoplasm. Because of their proximity to capillaries and nerves,[65,66] Robb[50] in particular suggested that their hormonal function began during the first half of gestation. He noted that islets gave staining reactions for both α and β cells in fetuses of between 10 and 13 weeks of gestation. It was also evident that the β cells tended to be arranged like the petals of a flower around a central capillary or capillaries, suggesting that secretion was either already occurring or was shortly to occur into the bloodstream. He also noted that this was not the case with regard to the α cells at this stage.

According to Liu and Trotter,[23] shortly before the acini begin to develop a few islands of Langerhans may be found on the outer surfaces of the ducts consisting of 2–4 nuclei embedded in deeply eosinophilic cytoplasm. Similarly, two types of islands of Langerhans were recognized in the fetus: the so-called primary and the secondary or permanent islands. The former gradually increased in size and then became detached from the ducts. By the third month some had attained a diameter of 35–40 μm. These were most numerous in the region of the body of the pancreas.

Capillaries soon penetrated these primary islands, and specific granules of various types were readily identified in the islet cells as soon as they differentiated from the ducts. In the larger islands, the β cells were most numerous. They occupied a central location in them and were closely associated with capillaries. The α and δ cells tended to be located at the periphery of the islands. By the fourth month the primary islands measured up to 45–55 μm, and by this time the β cells were centrally located and occupied about half of the total volume of these islands. The primary islands continued to enlarge during the fifth month due to proliferation of the α and to a lesser extent the δ cells. They noted that most of the primary islands began to degenerate during the sixth month, although a few continued to enlarge, and some measured up to 100 μm during the seventh to eighth month. Liu and Trotter[23] also noted that by this stage the vascular channels had disappeared. By birth, most of the primary islands had disappeared, although they were still observed in the pancreata of premature infants. Lymphocytic infiltration into the intralobular connective tissue was observed at the period of maximum degeneration.

Secondary islands of Langerhans were first noted during the fourth month and were often made up of an equal mixture of α and β cells. These islets gradually increased in number and volume,

often by fusion with adjacent groups of islet cells, and were also invaded by capillaries. At term, from the analysis of five stillborn infants, Ogilvie[67] estimated that these islands numbered between 105,014 and 486,486, with an average of 284,000. The larger islands measured about 75–95 μm in diameter and principally contained β cells. In Ogilvie's full study,[67] he analyzed 100 human pancreata (59 female and 41 male) varying in age from a number isolated from newborn individuals to one isolated from an adult of 64. After establishing the weights of these organs, he was able to calculate the following: (1) the weight of the acinar tissue; (2) the weight of the islet tissue; (3) the average weight of a single island; and (4) the total number of islands in a gland.

It is probably relevant to note that Liu and Potter[23] indicated that few others had reported the presence of primary and secondary islands. They were, however, convinced that they differed from each other not only in their time of appearance but also in their morphological appearance. They noted that the primary islands were relatively few in number and tended to have a more compact and concentrically layered appearance. The secondary islands consisted of irregular loosely arranged cords made up predominantly of β cells with only a few α cells randomly intermingled among them. They were also separated by wide, tortuous, vascular spaces. While the primary islands were first seen during the eighth week of development and developed from the primitive ducts of the pancreas, they reached their maturity during the fifth month with the majority degenerating thereafter. By contrast, the secondary islands were first seen during the third month and appeared to arise from the cells of the terminal ducts being located within the lobules among the acini. These, they believed, gave rise to the permanent islands.

In one study, a number of methods were used in an attempt to isolate pure islets from the human fetal pancreas at about 17–18 weeks of gestation.[68] In each of the methods used, the pancreata were isolated and minced. They were then divided into one of three groups and were exposed to either (1) no collagenase, (2) 5 minutes of collagenase digestion, or (3) 14 minutes of collagenase digestion. All preparations were retained in culture for 6–7 weeks and then were analyzed. In the 5-minute group, fragments with the appearance of islets were isolated but contained fibrous tissue and acinar duct elements not seen in islets. In the 14-minute group, islets were found that could be readily harvested from the cultures. These were morphologically similar to islets isolated from the intact fetal pancreas and contained cells that were positive for insulin and glucagon. They were also similar to those isolated from the neonatal rat pancreas. It was also demonstrated that these islets could be retained in tissue culture for at least 7–8 weeks.

The principal aim of another study published in 1985[69] was to try to obtain human β cells that might be used for transplantation into diabetic patients. The methodology used had been appropriately amended from that previously developed to isolate rat islets in culture.[70] Using this approach, human fetal β cells were successfully isolated and maintained in tissue culture. Fetal pancreata had been isolated from 37 consecutive prostaglandin-induced terminations of pregnancy of various gestational ages. After mild collagenase treatment, the partially disintegrated tissue was maintained in tissue culture medium supplemented with 20% fetal calf serum for seven days. This was to allow cell attachment and the outgrowth of the endocrine cells to occur. In 17 of 37 cultures, islet-like cell clusters were found and contained well-preserved pancreatic cells with large numbers of mitotic figures. A high rate of proinsulin biosynthesis and a small insulin response to secretory stimuli were noted. At the ultrastructural level, numerous cells containing granules were present, some of which were identified as β cells. Some of the harvested cells were transferred beneath the kidney capsule of nine nude (*Foxn1*^{nu}) mice. When these were euthanized 2 months later, seven contained grafts with islet-like cells present that contained insulin- and glucagon-positive cells.

In a study published in 1989,[71] pancreata were obtained from 18 terminations of pregnancy isolated at the 10th (n = 4), 12th (n = 7), and 14th (n = 7) weeks of gestation. These were analyzed histochemically for the presence of cells that immunoreacted with antisera raised against insulin (β cells), somatostatin (δ cells), glucagon (α cells), and pancreatic polypeptide (also termed F-type or PP-type cells). Such cells were found in each of the stages studied. At 10 weeks, these cells were all within or near to the epithelium of small excretory ducts. At the 12th week, some β cells were

also found in small clusters outside the ducts. At the 14th week, the β cells were in the form of large clusters close to the excretory ducts, and α cells were also represented. The β cells were the predominant type (about 50%) at the 10th week, the δ cells made up about 25%, the α cells about 15%, and the PP cells about 10%. By the 12th to 14th week, the β cells now represented about 36% and the PP cells about 6% of those present while the δ and α cells had increased to about 30% and 27%, respectively. It was calculated that the islets represented about 2, 6, and 21% of the total parenchymal volume of the pancreas at 10, 12, and 14 weeks, respectively.

ISLET TISSUE IN THE HUMAN ADULT

In the adult human, it is believed that the islet tissue represents only about 1–2% of the total weight of the pancreas, and numerous studies have indicated that individuals with diabetes generally have fewer islets than normal individuals. While there is general agreement about this point, the extremely rudimentary methodology used, even in the most recent means used to obtain these findings, has been much criticized over the years. In one of these studies,[72] it was established that there might be only one third to one quarter as many islets in diabetics as in nondiabetics. Similar reservations have also been expressed about studies that purport to provide information on changes in islet size, as the variation observed between normal individuals is so considerable.[72]

In Gepts's findings,[72] from his analyses of the pancreata of patients who died soon after the clinical onset of chronic juvenile diabetes, peri- and intra-insular inflammatory infiltrates were found in the islets of 68% of these individuals. The β cells were often completely absent from their islets, except in occasional cases, and the islets generally consisted of small atrophic cells. In some cases, he believed that during the preclinical phase of this condition an unknown, possibly extrapancreatic, factor exerted a strong stimulating action on the islet-forming capacity of the parenchyma, leading to a decrease in the number of β cells present. Once the condition was manifest, the islets consisted exclusively of α cells that were accompanied by atrophic tissue derived from the β cells. Even when β cells were present in the islets of these individuals, there was usually less than 10% of the normal expected number. All of these cells showed cytological signs of marked activity. The insular activity was not normal and appeared to have been produced by a small number of very hyperactive β cells.

Recent successes in treating type I diabetic patients with islet transplants have indicated that there might well be a need for an increase in the availability of islet tissue for this purpose. Studies have also indicated that multipotent stem cells within the pancreas are capable, under appropriate experimental conditions, of differentiating into functional islets.[73] When these cells are transplanted into diabetic mice, it has been demonstrated that this is capable of reversing insulin-dependent diabetes in these animals.[73] The establishment of methods of growing functional islet tissue from stem cells would seem to offer the possibility of treating individuals with diabetes in the long-term.

More recent studies clearly indicate that the pancreatic stem cells capable of differentiating into islet cells normally arise from an endodermally derived pancreas-specific precursor cell.[2] The transplantation of these cells to patients with type I diabetes has led to the relief of this condition for extended periods of time, and it is strongly recommended that this might be the most beneficial route for the management of this condition. The possible genetic mechanisms involved during the early stages of the development of the pancreas have also recently been discussed.

ENDOCRINE SECRETION OF THE ISLETS OF LANGERHANS

Numerous ultrastructural studies including several recent reviews have revealed that a number of distinct cell types have been identified in the pancreatic islets in a large number of mammalian species, each of which appears to possess a distinct function. While not distinguishable with the naked eye, the pancreas contains very considerable numbers of islets of Langerhans, and a number of studies have been undertaken over the years to estimate the number of islets present in the normal,

nondiabetic, individual (see above). Although large numbers are present, because of their relatively small volume, they still constitute a relatively small proportion of the gland. So much interest has been shown over the years on the topic of the pancreatic islets that at least several books have been devoted exclusively to these cellular complexes.[51,74]

The islets contain several thousand cells, and each is vascularized by one or usually more capillaries into which its hormonal secretions are liberated. The islets additionally contain a nerve supply from the coeliac plexus and from other sources, and these enter the gland with its arterial supply. It has been demonstrated that the nerves are both sympathetic and parasympathetic in origin, and fine, often unmyelinated, branches ramify throughout the gland. It has also been noted that many of the nerves initially innervate the acinar cells and then progress to innervate the islets.[75] It has also been noted that parasympathetic ganglia are located in the inter- and intra-lobular connective tissue and are frequently associated with insular cells to form neuro-insular complexes.[76,77]

Within the islets themselves, three types of nerve terminals have been reported.[78] Some are cholinergic (with 30–50 nm diameter agranular vesicles), others are adrenergic (with 30–50 nm dense-cored vesicles), and a third group have yet to be characterized (with 60–200 nm dense-cored vesicles). Curiously, in some cases more than one nerve terminal contacts a single cell.[79] Esterhuizen et al.[80] also revealed that there was both adrenergic and cholinergic innervation of both α and β cells in the islets of Langerhans of the cat.

To date, no overall hypothesis has been proposed to explain how the innervation of the pancreatic parenchyma and the islets functions. In animal studies it has been reported that sympathetic stimulation (from splanchnic nerves) of the islets diminishes the secretion of insulin, while parasympathetic stimulation (from the vagus) promotes secretion. The only mammal not known to have innervated islets is the spiny mouse, *Acomys cahirinus*,[78] while in the pigeon, no neural elements were found in the islet tissue.[77] It has also been demonstrated that stimulation of certain components of the vagus nerve stimulates insulin secretion whereas other components of the vagus nerve do not appear to do so.[80] This study demonstrated that stimulation of the right and left cervical vagus and the dorsal vagal trunk induced an immediate and significant elevation in immunoreactive insulin concentration in pancreatic effluent plasma whereas stimulation of the ventral vagal trunk did not. Curiously, the increases in immunoreactive insulin were not followed by decreases in blood sugar levels.

The general impression is formed that the autonomic nervous system does not play an important role in the functioning of the islet cells under normal conditions. However, stimulation of the vagus induces a limited degree of exocytosis and secretion of the cells associated with the pancreatic acini and small ducts. It is now generally accepted that nervous stimulation probably plays only a very limited role and that the secretory activity of the endocrine component of the gland is largely under hormonal regulation.

As glucose is the principal product of carbohydrate digestion, how it is utilized in the body as an energy source is of critical importance and this is largely controlled by the action of the endocrine secretions of the pancreas. Insulin, for example, secreted by the β cells of the islets, is produced from preinsulin. The latter is assembled on the ribosomes of the endoplasmic reticulum, and this substance is then converted to proinsulin. This is then transferred to the Golgi apparatus in the form of small vesicles, although no insulin appears to be present within the Golgi complexes. The possibility has been proposed that the proinsulin is then converted to insulin in small vesicles that bud off from the trans-Golgi cisternae.[11,12]

For reasons that are not yet understood, not all of the insulin is secreted. Insulin secretion appears to be stimulated by the elevation of blood glucose. The insulin released into the circulation diffuses throughout the body and bonds to appropriate insulin receptors, which facilitates the entry of glucose into the cytoplasm of most of the cells of the body.

At the ultrastructural level, it has been noted that the islet cells possess considerably less granular endoplasmic reticulum than that present in the acinar cells. The small vesicles and secretory granules present in the cytoplasm are usually located close to the perinuclear Golgi complex. The only way of distinguishing among the different islet cell types is by careful analysis of the size, density,

and internal structure of their secretory granules. With regard to the α cells, the central region of their granules is extremely electron dense, while their outer zone tends to be of low density. By contrast, the β cells tend to be quite distinctive. In many species they contain one or more dense crystals within a low-density matrix. The latter is often extracted in tissue preparation, and the crystals then tend to stand out against what appears to be a clear background. In those species where no crystals are present, their β cells may be difficult to distinguish from α cells. In all of the islet cells, the contents of the secretory granules are released by exocytosis into the extracellular space when it either acts on neighboring islet cells or enters the blood through pores in the neighboring capillaries.

In a recent study, Beringue et al.[81] hypothesized about a relationship between peripheral glucose intolerance in adults born with intrauterine growth retardation (IUGR) that might involve insulin resistance as a consequence of abnormal endocrine pancreas development during their fetal life. They analyzed the insulin-containing cells in 21 fetuses with IUGR and compared these with the pancreata in 15 control fetuses. Pancreata from fetuses older than 32 weeks (mean age of 36 weeks in both groups) were analyzed histologically. All genetically abnormal fetuses were excluded from this study. Six intermittent sections from each pancreas were immunostained with anti-insulin antibody, and the results were analyzed using computer-assisted morphometry. Islet density was similar in the two groups, as was the percentage of β cells located within the islets. This study suggested that there was probably no developmental abnormality in these individuals and further indicated that peripheral insulin resistance was the most likely mechanism leading to glucose intolerance in these adults.

DEVELOPMENT OF THE PANCREAS IN THE RAT

In one of the most comprehensive studies on the development of the pancreas in the rat, Hard[44] isolated embryos from Wistar strain rats at approximately 24-hour intervals from the 11th day of gestation to birth (22 days). Two litters were isolated at 20 days and at birth, while postnatal observations were made on pups isolated at the following times after birth, 24, 48, and 72 hours and at 4.5, 7, 9, 13, and 15 days after birth. Whole embryos, up to the 16th day of gestation, were isolated and fixed, and from this stage onward the abdominal cavity was first opened and entire embryos (or fetuses) were placed in fixative. The desired tissues were then dissected out following their fixation. Hard[44] first observed β granules in the islet cells in the rat fetus within a few hours of the first appearance of capillaries within the islets but believed that the β cells were probably storing their granules rather than actively secreting their contents.

In a particularly interesting study, an isolated region of the primitive duodenum of the rat was maintained in a simple tissue culture system.[82] In this system this region of the primitive gut gave rise to a number of differentiated organs, including a dorsal and a ventral pancreas. The two pancreata that formed did not fuse, thus allowing independent analyses to be undertaken of the two lobes so that they could be compared with in vivo findings. It was noted that the dorsal pancreas was the first to appear at the equivalent of the 23–25-somite stage, while the ventral pancreatic rudiment first appeared approximately 12 hours later at the equivalent of the 29–30-somite stage. Both pancreata exhibited identical patterns of morphological and biochemical differentiation, and both contained the same exocrine enzymes and hormones at similar levels. The only obvious difference was that the dorsal pancreatic rudiment possessed a five-fold higher level of glucagon activity.

The endocrine cells located in the duct system of the pancreas and in the biliary duct system of adult rats were examined in an attempt to establish their possible roles in these locations.[83] Their initial appearance was noted as was their developmental progress at different stages during fetal and postnatal life. The earliest insulin, glucagon, and cholecystokinin (CCK) cells were recognized at 12 dpc in the pancreatic primordium. By 14 dpc, however, glucagon and CCK cells were first recognized in the epithelial lining of the common hepatic and hepatic ducts and remained the dominant endocrine cells during the fetal period in these locations. Insulin and pancreatic polypeptide cells were first recognized in the common hepatic duct on 16 and 18 dpc, respectively. Somatostatin cells were not recognized in the hepatic duct system during the fetal period, being first seen at about 7

days after birth. The somatostatin, CCK, and pancreatic polypeptide cells increased while glucagon and insulin cells gradually decreased in number up to the adult stage. The somatostatin cells were the commonest of the cells seen in these ducts in the adults. It was proposed that their possible role was in influencing the exocrine secretion as well as the drainage of the pancreatic and bile fluid.

Dixit et al.,[84] in an interesting paper, determined the insulin content of microdissected islets obtained from both normal and alloxan-induced diabetic mothers given this agent on the 10th day of pregnancy and from normal postnatal rats up to 5 weeks of age. They noted that the rat fetuses isolated from the alloxan-induced diabetic mothers showed decreased insulin content compared with the controls. It was suggested that this difference might have been attributed to a difference in maternal blood glucose,[60] as these females displayed severe hyperglycemia in excess of 250 mg per 100 ml. Others had demonstrated[63] that markedly elevated blood glucose levels led to degradation of the fetal islets with low insulin content.

In another study, maternal food restriction (65% restriction of normal food intake) was carried out at three different periods of gestation to investigate whether this had an effect on the endocrine pancreas of the fetus.[85] The three periods of food restriction studied were (1) from 0 to 7 days of gestation, (2) from 7 to 14 days of gestation, and (3) from 14 to 21 days of gestation. While the maternal body weight was reduced in groups 2 and 3 compared with controls, no other changes in basal parameters were noted in any of the groups. However, body weights of fetuses were decreased in all three groups, while pancreas weight was reduced only in the group 3 fetuses. An increased insulin islet content and hypertrophy of β cell mass was noted. These findings suggest that the development of the fetal pancreas depends on a balanced maternal glucose intake throughout pregnancy and that undernutrition, particularly during the last third of gestation, may induce alterations of the fetal endocrine pancreas.

In another study in which food was restricted during late pregnancy, a decrease in β cell mass was observed in the offspring at birth. If prolonged maternal malnutrition until weaning occurred, this led to an irreversible decrease in β cell mass in the adult male offspring. Under control conditions, the maternal endocrine pancreas demonstrated an acute and reversible increase in β cell mass. In a subsequent experiment, the effect of pregnancy on 4- and 8-month-old female rats malnourished during their perinatal life was compared with age-matched controls.[86] The 4-month-old females showed a normal glucose tolerance but a significant decrease in insulin secretion during oral glucose tolerance tests. They also doubled their islet mass during late gestation. In the 8-month-old experimental group, their insulin content during pregnancy was reduced to half that of controls, and they did not display the characteristic increase during gestation observed in the pregnant control females. In the experimental animals, the islet mass remained throughout pregnancy at the nonpregnant level. This study therefore concluded that early malnutrition had dramatic consequences on the ability of the endocrine pancreas to meet the increased demands of pregnancy in the aged population.

In a more recent study, the diets of pregnant and lactating female rats exposed to a low protein diet were supplemented with taurine during both their pregnancy and their period of lactation.[87] This had an interesting influence on the pancreatic islets of their offspring. Protein deprivation induced abnormal development of the islet cells, with a reduction in their β cell mass and islet cell proliferation, as well as an increase in the incidence of islet cell apoptosis. Boujendar et al. also noted a decrease in maternal and fetal taurine in these animals. This study had been undertaken to investigate the role of taurine in the offspring of these rats by supplementing the protein deficient diet of their mothers with taurine until day 30 after delivery. β and other islet cells were determined as well as the incidence of apoptosis in the islets during their early postnatal period. It was noted that despite the maternal protein deficiency, adequate β and other endocrine cells were present in the islets of the taurine-exposed pups. It was concluded that taurine supplementation in their low protein diet prevented the abnormal development of the endocrine pancreas previously observed in the offspring of protein-deficient females.

Low birth weight infants and poor growth in early life may predispose to noninsulin-dependent diabetes. These conditions were induced when fetuses were exposed to either pre- or postnatal protein deprivation or to deprivation during both of these periods.[88,89] Following maternal protein

deprivation during *both* periods, and deprivation during the postnatal period, the offspring had fewer but larger islets than in the control group, while those exposed to maternal deprivation during the prenatal period alone had more numerous but smaller islets. Offspring from all of the groups studied possessed more irregularly shaped islets and a reduction in the amount of β cells within each islet. These findings indicate that major structural changes may result in size, shape, and composition of the islets in the offspring following a reduction in maternal protein intake during and shortly after pregnancy and also support the theory that events occurring during the early development of the pancreas may influence the situation in the adult.

MORPHOLOGICAL FEATURES OF THE PANCREAS IN THE ADULT RAT[90]

The color of the adult pancreas in the rat tends to be a whitish gray. It weighs between 550 mg in a 100 gm rat to closer to 1 gm in a 300 gm rat.[91] The gland is very lobulated and usually extremely difficult to distinguish from the adipose tissue that surrounds it, although it is usually slightly darker in color and has a somewhat firmer consistency. While it is located in the upper part of the peritoneal cavity, its left lobe runs along the dorsal aspect of the stomach and greater omentum, and, as in the human, it runs along with the splenic artery toward the hilum of the spleen. Its right lobe and body are principally apposed to the left side of the mesoduodenum and the beginning of the mesojejunum. The majority of the length of the hepatic duct is surrounded by pancreatic tissue.

The gland is said to contain between 15 and 40 excretory ducts.[90] These fuse to form a minimum of two main ducts but may form as many as five to eight main ducts. These open into the common bile duct at various levels along its course. The first and largest of the collecting ducts is always from the left lobe and is termed the splenic duct. Occasionally, relatively small ducts enter the duodenum directly.

On the basis of the distribution of its various ducts and other anatomical features, such as its vascular supply, it has been suggested that the rat pancreas should be divided into splenic, duodenal, gastric, and parabiliary segments.[91] Based on these subdivisions, it has been suggested that various operations may be performed, such as total or segmental partial pancreatectomy, ligation of the major duct from, for example, the splenic segment, or ligation of the major arterial supply to the splenic segment. The wet weight of each of these subdivisions also gives a linear relation to the total body weight. It is suggested that because of the relative ease of subdividing the gland into defined segments, the rat may provide a reasonable model for undertaking repeatable procedures involving segmentation of the pancreas, as required in certain experimental studies.

It had been noted previously that in this species diabetes would ensue only if 99–99.5% of the pancreas was removed but not if only 90–95% was removed.[92,93] This observation emphasized that for experiments in which *total* pancreatectomy was required, it was essential that the meticulous removal of even small amounts of pancreas had to be carried out. If 99–99.5% of the pancreas was successfully removed, at 19 hours after the operation the rats had hyperglycemia, glycosuria, ketonuria, gastric retention fluid, and large amounts of fat in the liver and in the renal tubules. If not treated with insulin, they died within 48 hours in diabetic coma.[92] Uram et al.[93] noted that the rat is capable of normal or near normal digestion with less than 1% of the contribution of its exocrine pancreas. Similarly, they also demonstrated that the ability of the duct-ligated rat to reestablish exocrine flow was demonstrated and that a procedure was developed to circumvent this adaptation.

At the histological level, the pancreas is a compound acinar gland with relatively little interstitial tissue. The acini vary considerably in size and shape. The individual lumen tends to be narrowed due to the presence of large numbers of microvilli. The canaliculi branch from the main ducts within the gland and end blindly. The excretory fluid is secreted into the small diameter ducts and then passes into the larger diameter drainage ducts. The arrangement of the duct system within the pancreas has been studied using scanning electron microscopy.[90,94]

In a study undertaken to determine the number of islets present in the rat pancreas, considerable differences were reported both between strains, and in the pancreata isolated from individuals of different ages. According to Hellerström,[70] their number ranged from about 4,000 to 24,000, and

the period of most rapid growth in number occurred in the perinatal and early postnatal period.[95] It should be noted that these figures were substantially greater than those reported previously by Hellman,[55] where the number present in the newborn was 243± 21 and in older rats was 3320± 184 (see above). Age-related changes in islet function have also been noted in the rat.[96] It had been noted, for example, that aging rats became less tolerant of glucose as they increased in age, despite an increase in the number of large islets present.[97] Despite these earlier findings, these age-related changes in islet function were not confirmed in more recent studies on islet function,[98,99] and no age-related impaired efficiency in islet function was noted.[100]

GENERAL OBSERVATIONS ON THE PANCREAS IN THE MOUSE

Figures 6.1 through 6.14 illustrate the development of the mouse pancreas. Few relatively early studies have been undertaken on the morphological, histological, or ultrastructural analysis of the mouse pancreas. This should not be altogether surprising because most of the early workers in this field noted that there were few species differences between the rat and the mouse. Consequently, they undertook numerous experimental studies on the rat, principally because of its increased size and the greater accessibility of the pancreas in this species. Of the few relatively early studies on the mouse pancreas, the two by Munger are particularly helpful: One analyzed cellular differentiation in the pancreatic acinar cells,[101] while the other analyzed the ultrastructure of the pancreatic islets.[103] In both of these studies, details were provided on the development of these components of the pancreas, using phase contrast and electron microscopy and light and electron microscopy, respectively. By contrast, relatively recently a substantial number of genetic studies have been carried out in inbred strains of mice, particularly involving the analysis of pancreatic function in strains of mice with hereditary diabetes and obesity syndromes.[100]

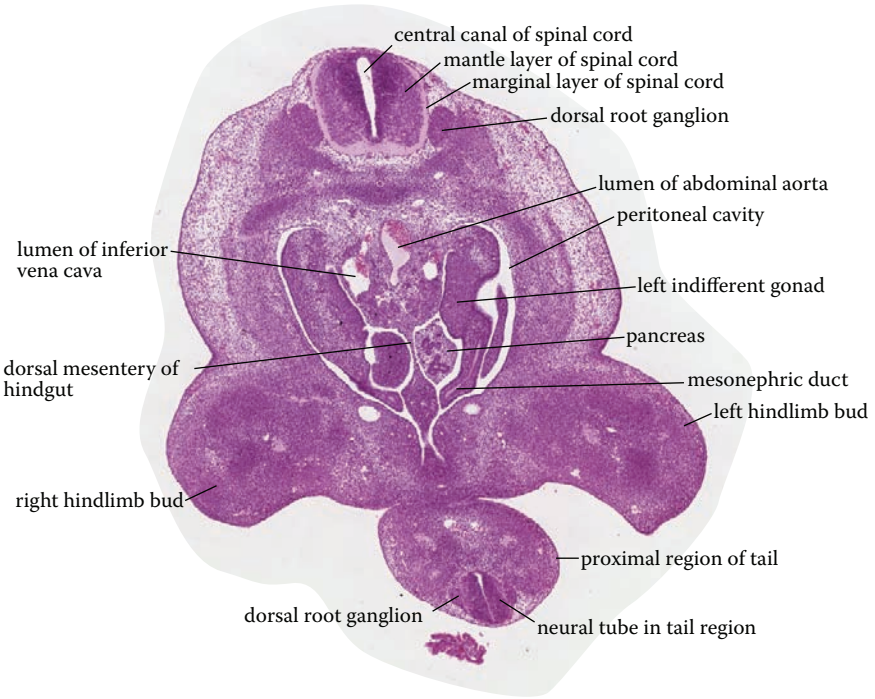

FIGURE 6.1 (E11.5_Fig6.1.svs) Low magnification, transverse section through the mid- to lower abdominal region of a mouse embryo (E11.5–12) that is stained with hematoxylin and eosin (H&E). Part of the left caudal region of the pancreas, close to the dorsal mesentery of the hindgut, is seen in this section. Note that this region of the pancreas is located close to the left indifferent gonad and the left mesonephric duct.

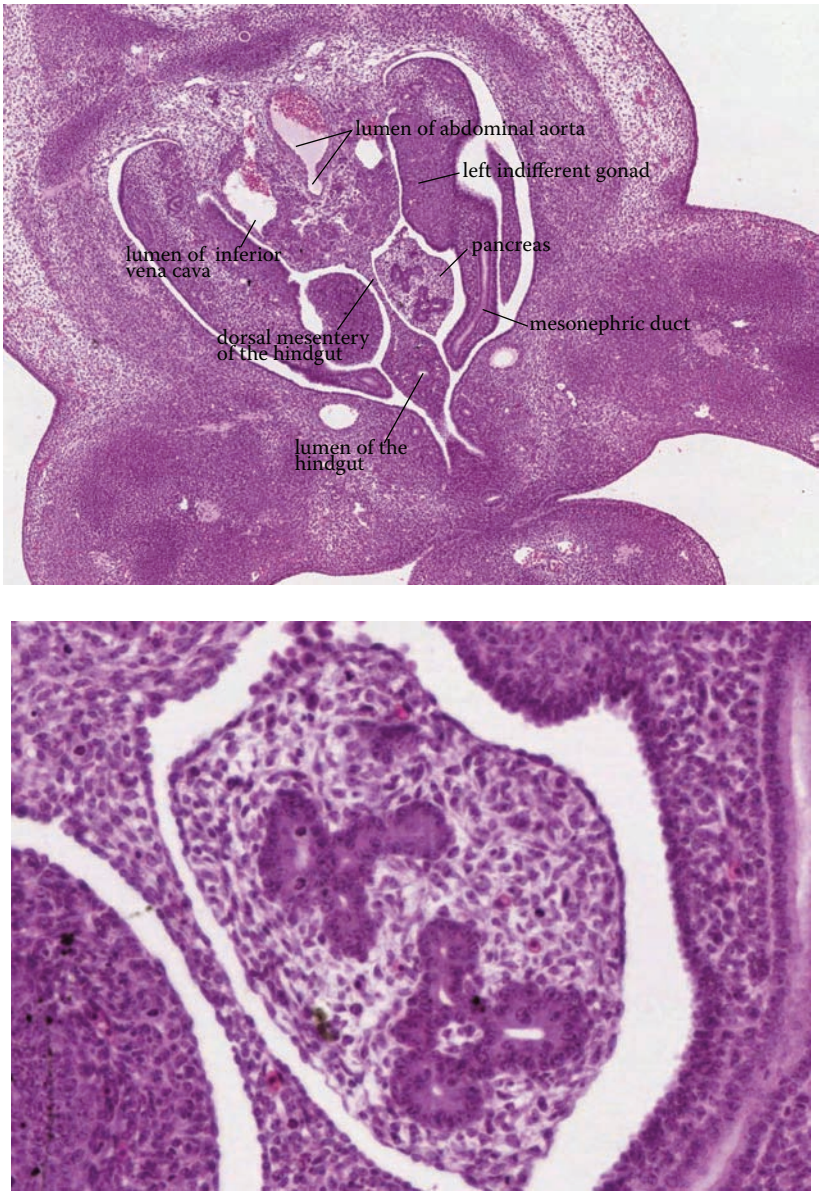

FIGURE 6.2 Medium (top) and high (bottom) magnification of Figure 6.1 illustrates the caudal region of the pancreas. This emphasizes its proximity to the dorsal mesentery of the hindgut, the left indifferent gonad, and the left mesonephros and its duct.

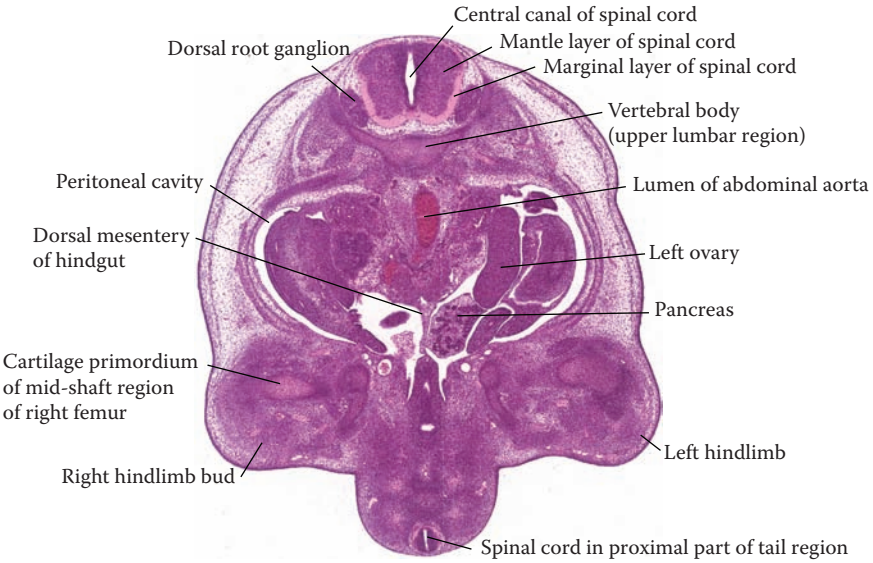

FIGURE 6.3 (E12.5_Fig6.3.svs) Low magnification, transverse section through the lower abdominal region of a female mouse embryo (E12.5–13) that is stained with H&E. Part of the left caudal region of the pancreas is located close to the dorsal mesentery of the hindgut and anterior (inferior) pole of the left ovary.

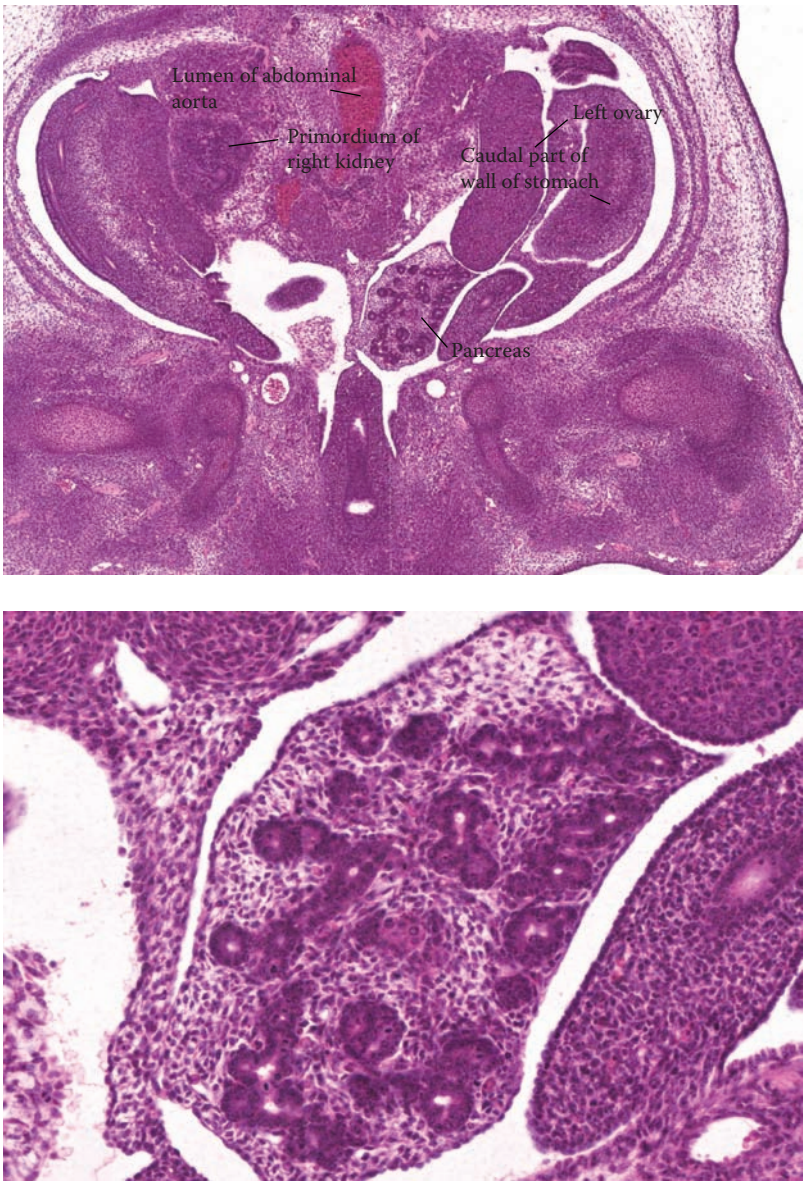

FIGURE 6.4 Medium (top) and high (bottom) magnification of Figure 6.3 shows the caudal region of the pancreas. Early stages in the differentiation of this organ are seen.

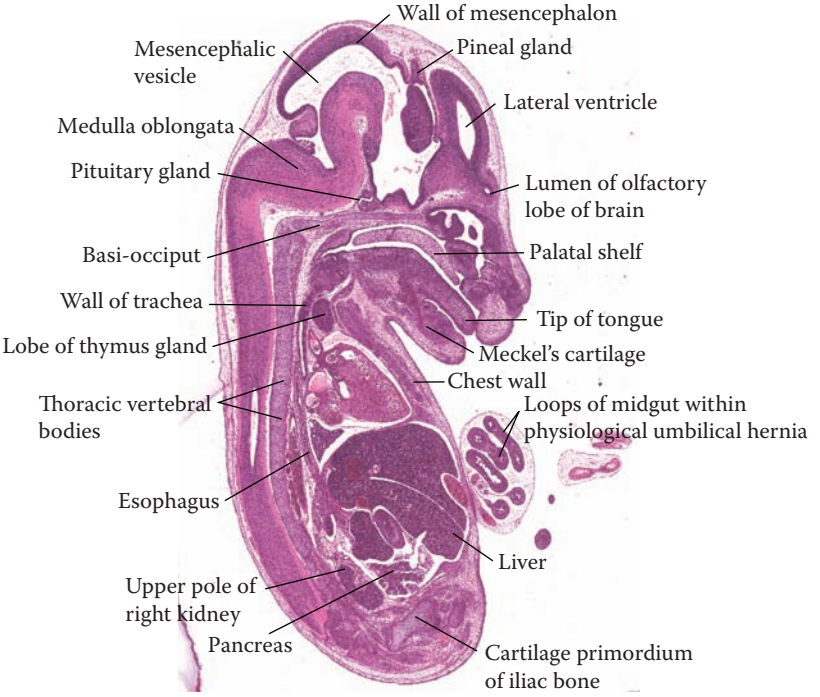

FIGURE 6.5 (E14.5_Fig6.5.svs) Low magnification, right sagittal section through the abdominal region of a mouse embryo (E14.5) stained with H&E that clearly displays the caudal region of the pancreatic primoridium, and its proximity to the right kidney. The wall and contents of the physiological umbilical hernia are also clearly seen.

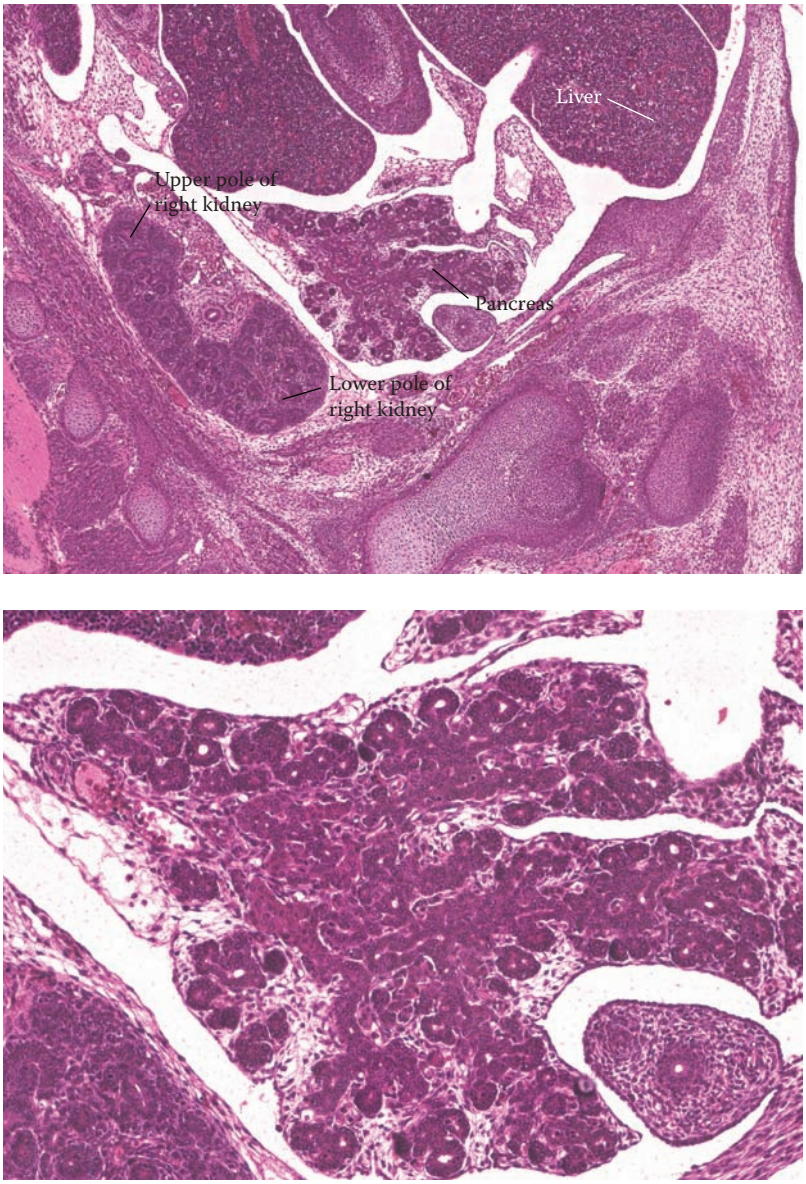

FIGURE 6.6 Medium (top) and high (bottom) magnification of Figure 6.5 presents a view of the pancreatic primordium. Note that an increased degree of differentiation of the duct system is now seen compared with that observed at earlier stages of the differentiation of this organ, with early evidence of islet formation.

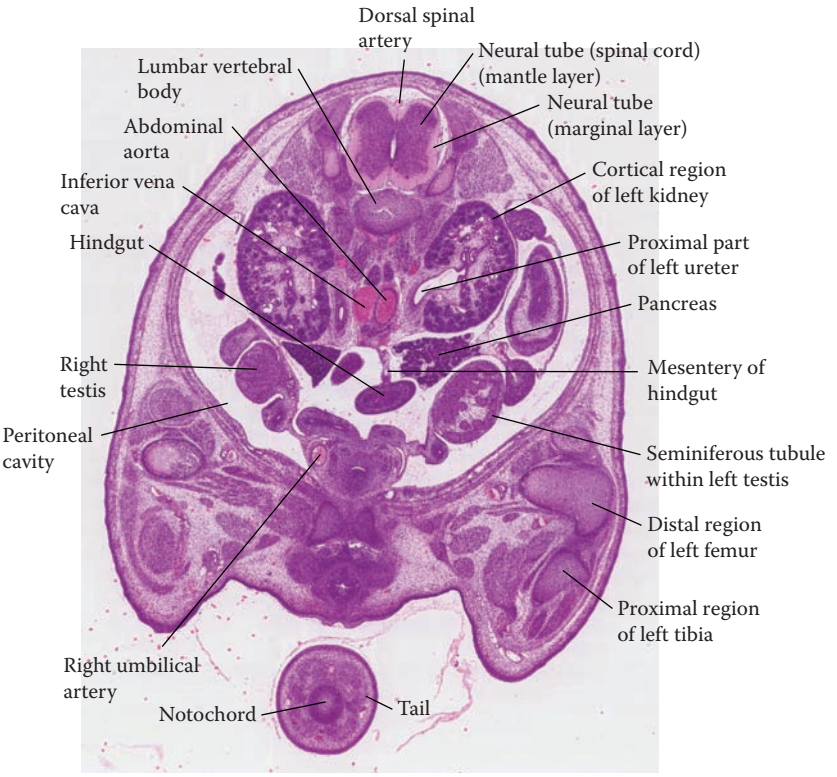

FIGURE 6.7 (E15.5_Fig6.7.svs) Low magnification, transverse section through the midabdominal region of a male mouse embryo (E15.5) stained with H&E that clearly displays the proximity of the future tail region of the pancreas to the lower pole of the left kidney. Its anterior part is seen to be in proximity to the caudal part of the wall of the left testis, while its tail region is directed toward the lower part of the stomach and hilar region of the spleen (not seen in this section). Medially, the region of the pancreas seen in this section lies in proximity to the left side of the hindgut and its dorsal mesentery.

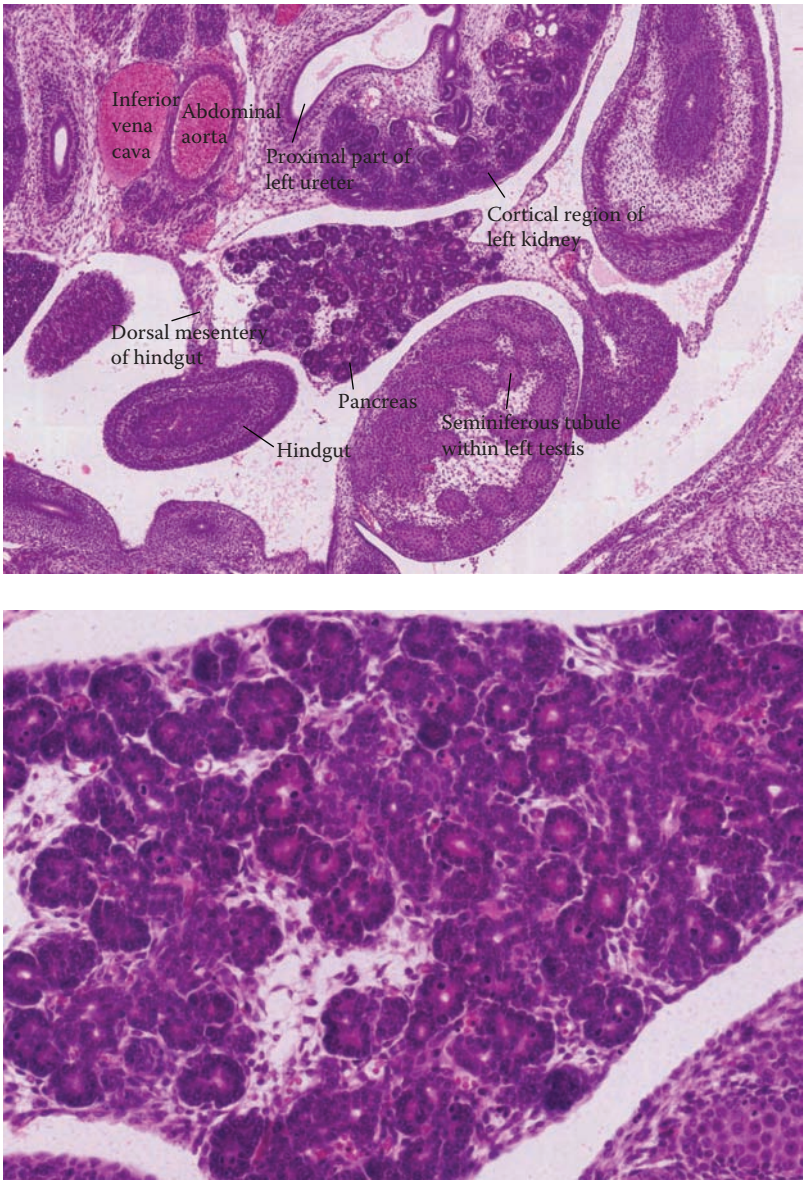

FIGURE 6.8 Medium (top) and high (bottom) magnification of Figure 6.7 shows the pancreatic primoridium. Also note the increased degree of differentiation of the organ compared with that observed at earlier stages of its development. The detailed histological morphology of the testis at this stage of development is also seen.

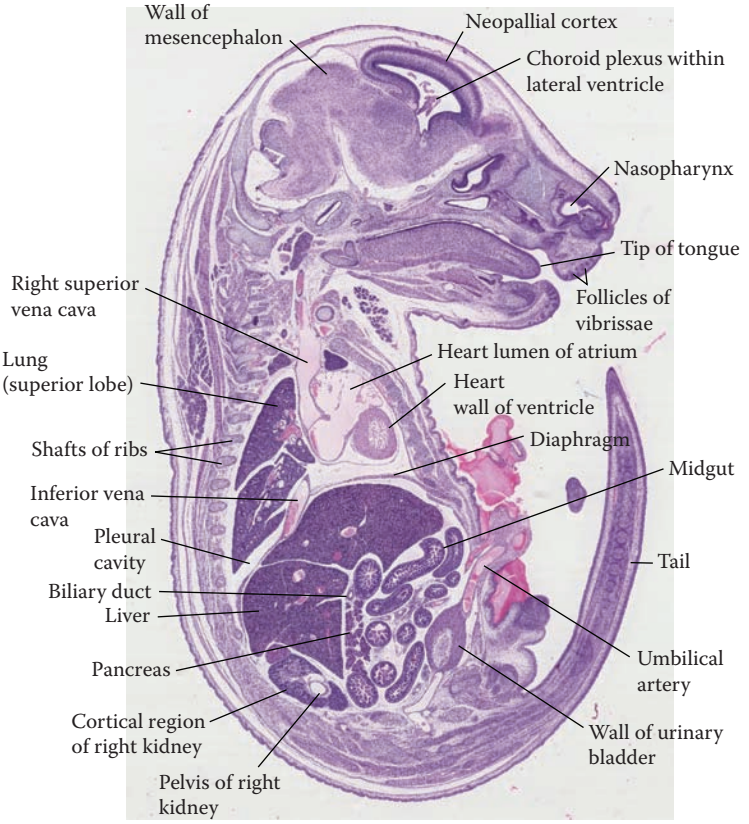

FIGURE 6.9 (E16.5_Fig6.9.svs) Low magnification, right sagittal section through the abdominal region of a mouse embryo (E16.5) stained with H&E that displays a small part of the pancreas located in proximity to the anterior, inferior surface of the lower lobe of the liver. The most rostral part of the pancreas is located close to parts of the biliary apparatus, while other parts of this organ are located close to various regions of the gut and to the anterio-inferior pole of the right kidney.

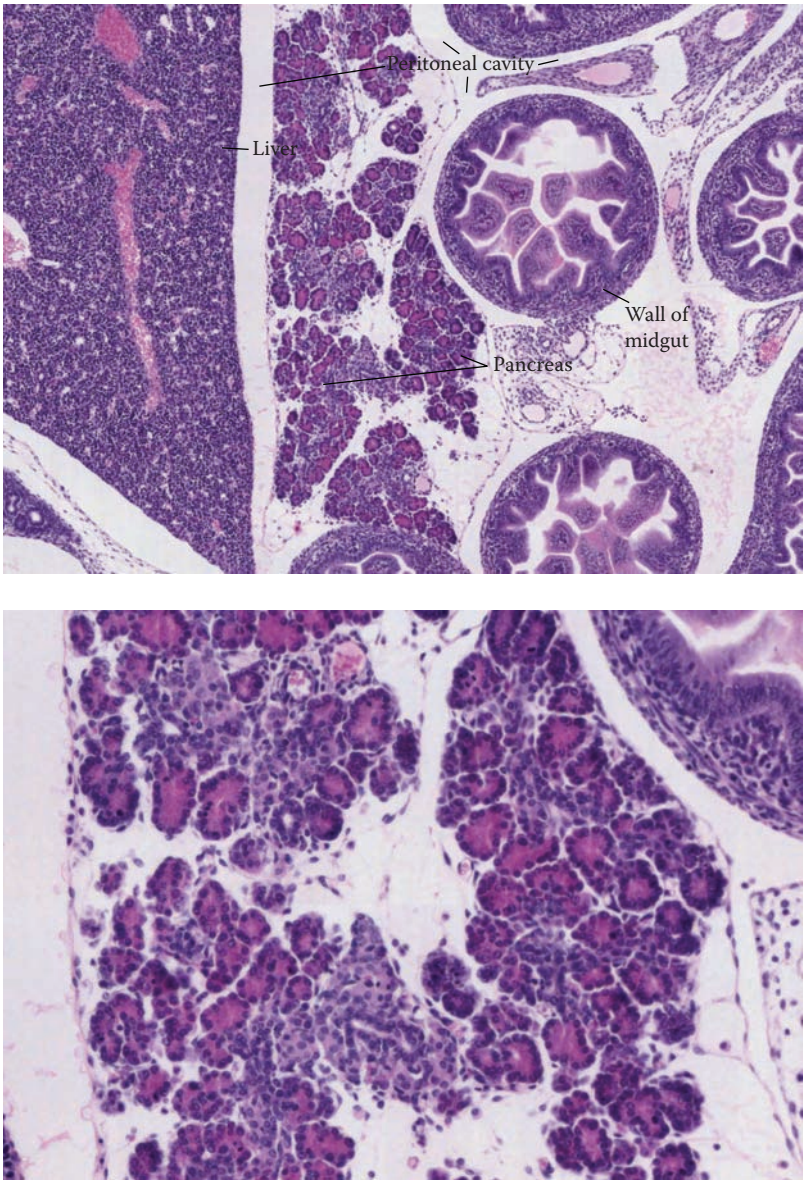

FIGURE 6.10 Medium (top) and high (bottom) magnification of Figure 6.9 illustrates the middle region of this part of the pancreas. Note again the increased degree of differentiation of this organ compared with earlier stages of its development. The detailed histological morphology of a typical section through the midgut region is also seen in this section.

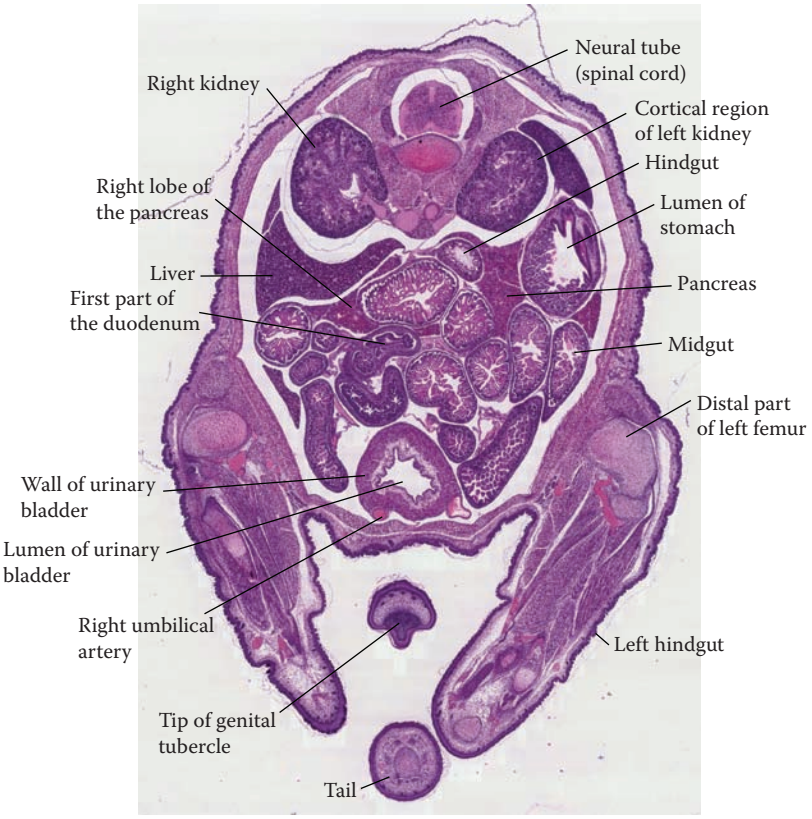

FIGURE 6.11 (E17.5_Fig6.11.svs) Low magnification, transverse section through the midabdominal region of a mouse embryo (E17.5) stained with H&E. This section displays lower poles of the right and left kidneys, the duodenum and the lumen and wall of the middle region of the urinary bladder, as well as various regions of the midgut and hindgut. Note that regions of both the right and left parts of the pancreas are present in this section.

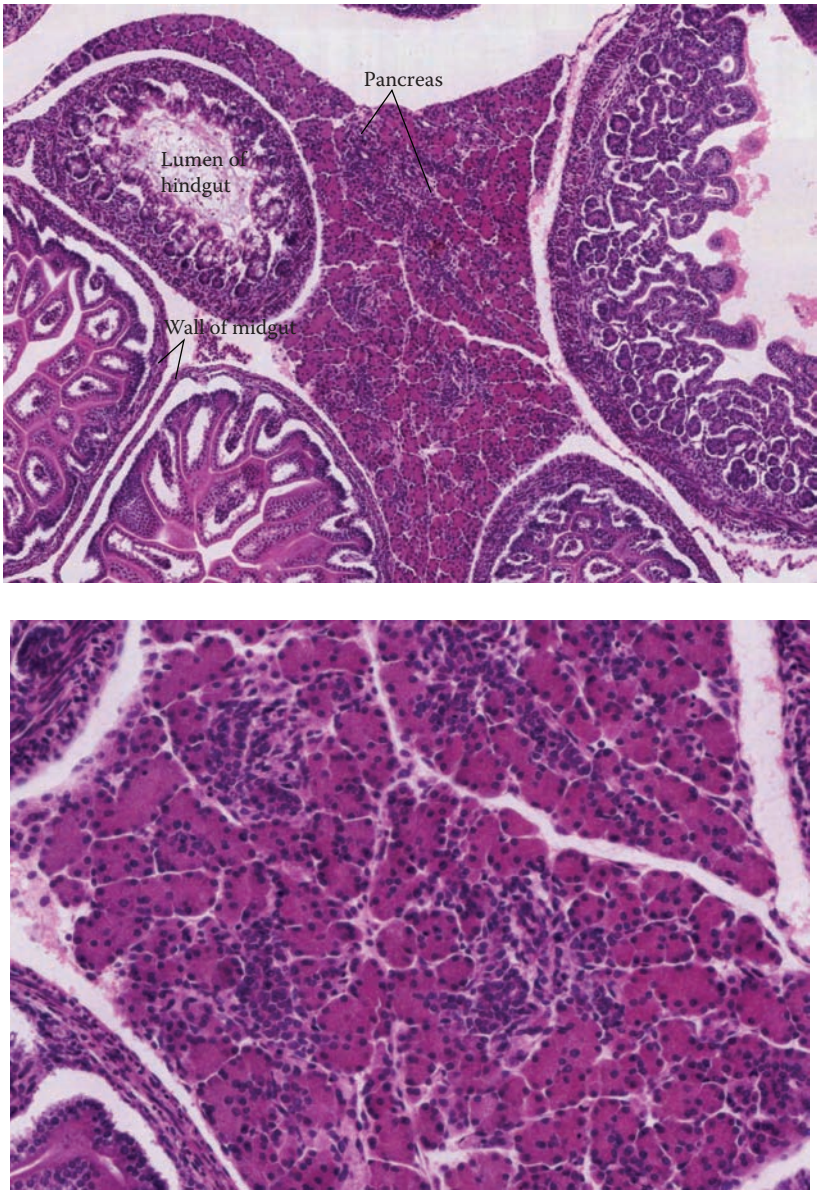

FIGURE 6.12 Medium (top) and high (bottom) magnification of Figure 6.11 shows part of the left lobe of the pancreas with its proximity to a region of the hindgut in this location. Sections through parts of the midgut are also displayed in the medium magnification image. The duct system of the pancreas appears to be well differentiated at this stage, although the location of the islet regions is not readily observed.

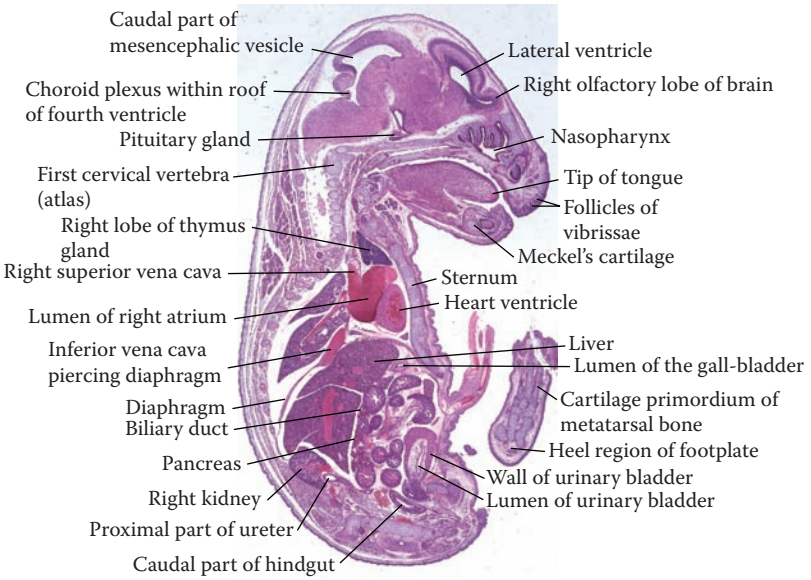

FIGURE 6.13 (E17.5_Fig6.13.svs) Low magnification, sagittal section just to the right of the medial plane of a mouse embryo (E17.5–18) stained with H&E. This displays most of the abdominal viscera. The two lobes of the liver as well as the right kidney and wall as well as the lumen of the urinary bladder are clearly seen, as is the caudal part of the hindgut. Only a small part of the right lobe of the pancreas is displayed in this section, as is the lumen of the gallbladder.

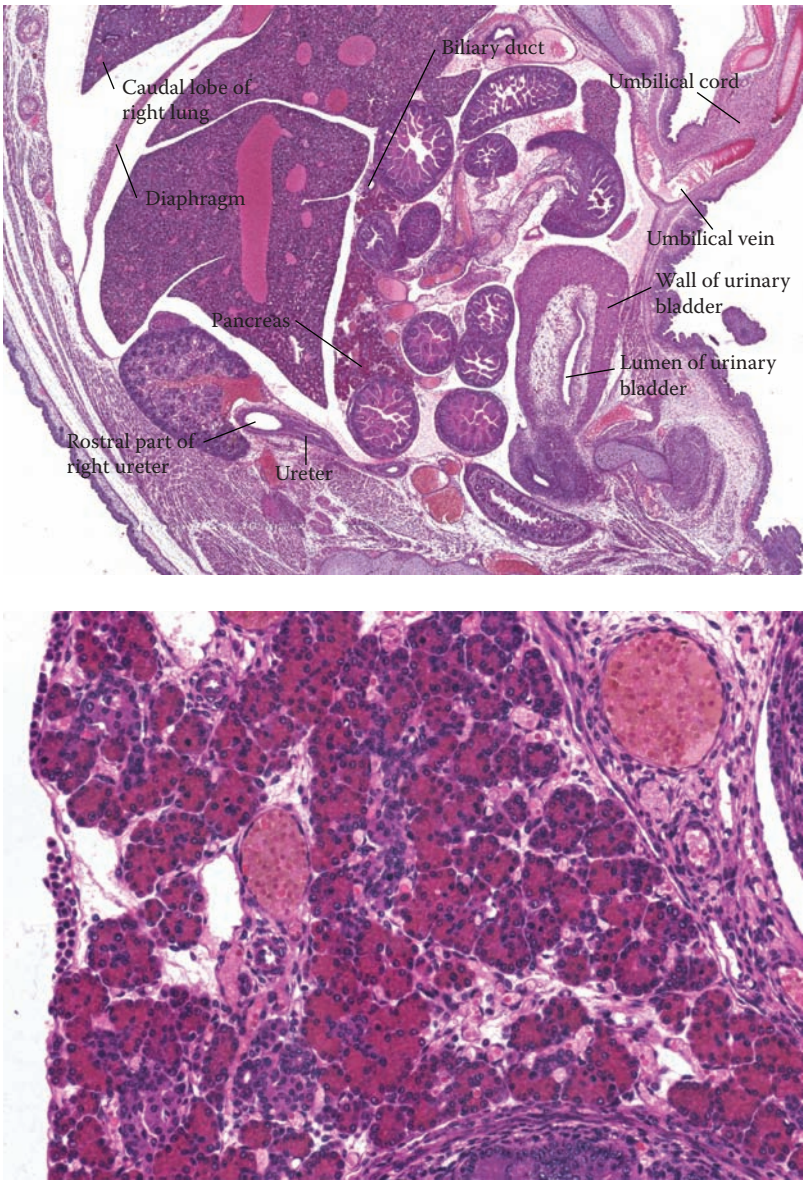

FIGURE 6.14 Medium (top) and high (bottom) magnification of Figure 6.13 illustrates a small part of the right lobe of the pancreas.

GROSS MORPHOLOGY OF THE PANCREAS IN THE MOUSE

The pancreas in the mouse is a relatively diffuse pale pink structure. It occupies the upper region of the abdomen, being bounded by the C-shaped duodenum to the right and the hilar region of the spleen to the left. It is principally located behind the stomach, being entirely retroperitoneal, and is divided into irregular lobes and lobules. Like the situation in the rat, it possesses various numbers of excretory ducts. While some enter the bile duct, at least one or more enters the duodenum close to the site of entry into it of the common bile duct. A loose connective tissue capsule also surrounds the pancreas.[103] As in all the mammalian species discussed previously, the pancreas in the mouse has an exocrine and endocrine function. The principal emphasis in the following section is on the embryological development of the islets of Langerhans and some of the studies that have been undertaken to investigate their role in this species.

The islets present in the adult mice were in many respects very similar to those observed in the pancreata of adult rats. Both displayed α and β cells, with the β cells principally confined to the central region of the islets and the α cells generally located toward the periphery. Small capillaries were also present in close proximity to all of the islets. Both of these cell types were readily recognized because of their different ultrastructural features.

DEVELOPMENT OF THE ISLETS DURING THE PRE- AND EARLY POSTNATAL PERIOD

The developmental studies reported by Munger on the differentiation of the islet cells[101,102] were based on the analysis of three to five mouse embryos isolated on days 13, 15–18, and 20 of gestation, and similar numbers of mice were isolated on postnatal days 1, 2, 4–7, 9, 11, 12, 19, and 21. Pieces of pancreatic tissue were isolated and fixed for light microscopy, sectioned at a nominal thickness of 5 μm following their embedding in paraffin, and stained by a variety of means. Other samples were fixed in osmium, embedded in plastic, and then sectioned at 1 μm for examination by phase contrast microscopy. This was to identify the islet tissue. Thin sections were then cut of the islet tissue for examination by transmission electron microscopy at initial magnifications of between 3,000 and 9,000.

When the pancreatic samples from the 13 dpc embryos were examined, it was not possible to recognize either α or β cells, although it was believed that the undifferentiated cells present were probably their stem cell precursors. It was, however, believed that a few of the β cell precursors were recognized by 15–16 dpc, and it was thought that primitive islets were recognized at the light microscopic level at this time. These were principally located adjacent to the ducts. At the ultrastructural level, a few of the cells adjacent to the pancreatic ducts were believed to represent immature β cells. The Golgi apparatus in these cells was particularly prominent. Cilia were also occasionally recognized in some of these primitive β cells. Primitive islets were recognized by about 18 dpc, although they possessed no α cells. The islets were readily recognized because of their typical islet arrangement.

By 18–20 dpc, these islet cells contained typical β granules, being very similar to those seen in adult β cells. Numerous small capillaries were also present at this stage, being located in close proximity to the islets. In the early postnatal material, the islet cells closely resembled those seen in the adult, although it was still not yet possible to recognize the α cells. At the margins of the islets, it was believed that the undifferentiated cells present probably represented primitive α cells. By 7 days after birth, it was now possible to recognize α cells at the periphery of the islets, and these were intermixed with poorly or occasionally nonstaining cells believed to represent immature α cells. By this stage almost all of the β cells present appeared identical to those seen in adult material. By 2–3 weeks after birth, the islets were identical to those seen in adult material. It is relevant to recall here that Grillo had previously revealed the presence of insulin or an insulin-like substance in the

mouse embryo at 16 dpc.[54] This finding suggested that this substance was present before mature β granulation could be detected microscopically.

Light microscopical observations associated with a variety of standard staining techniques and aided by histochemistry and autoradiography subsequently confirmed the presence within the pancreatic islets of β cells in the mouse on the 14th day of development.[51,104] In a later study, using conventional biochemical and histological methods associated with DNA flow-cytometric analysis the postnatal development of the mouse pancreas was studied over the period between birth and day 90. It was suggested that the pattern of development was biphasic.[105] The first phase extended from birth to day 15 and was dominated by the growth of the endocrine cells of the pancreas, as most islets achieved adult form by day 15. The proliferation and growth of the exocrine pancreas dominated the second phase, from days 15 to 30. Maximum numbers of the latter cells were seen by about day 20. This study suggested that in the mouse, the optimal time to isolate islet cells was toward the end of phase 1 and before the onset of phase 2. Similarly, the optimal time for isolating exocrine cells for growth in culture was likely to be during the early part of phase 1, between days 5 and 9, before most of the islet cells had attained their adult form.

STUDIES EXCLUSIVELY INVOLVING THE ENDOCRINE COMPONENT OF THE PANCREAS

Because aging is believed to be an important factor in noninsulin-dependent diabetes mellitus, it was proposed that the cells within the islets of mice of increasing age—at 3, 9, and 30 months—should be studied.[106] Probes for insulin, glucagon, somatostatin, glucose transporter-2 (GLUT2), glukinase, elastase-1, and beta-actin were studied by slot-blot analysis of cellular RNA isolated from the pancreas of these mice. A progressive age-dependent decrease in insulin mRNA levels (this decreased by 40% with age) and paralleled decreases in GLUT2 were observed. It was also noted that glukinase mRNA increased markedly, somatostatin and glucagon mRNA levels remained fairly static, while levels for elastase-1 and beta-actin increased with age. It was suggested that these findings indicated a progressive decline in the activity of the endocrine pancreas with age. This appeared to principally affect the β cells and not the α or δ cells, and it was suggested that this might reflect an age-dependent risk of the development of diabetes.

In an attempt to study the development of type 1 diabetes, mice were exposed to multiple low-dose administrations of streptozotocin. This was to investigate the fate of the α cell population of the islets during and after this period. Adult male mice were injected with this agent over a 5-day period, and the development of hyperglycemia was monitored over the following 28 days. A morphometric analysis of their islet endocrine cells was then performed.[107] A reduction of islet cell area was observed following two injections of streptozotocin, and a further 35% reduction occurred during the rest of the study. Hyperglycemia was noted from day 7 onward. A 2- to 3-fold increase in α cell population was observed, with a highest value observed on day 21 paralleled by high secretion activity of these cells at this time. It was suggested that multiple doses of this agent caused the disappearance of the β cell component of the islets, with a compensatory expansion of the α cell population.

Various methods have recently been employed to investigate both the development and differentiation of pancreatic islet cell components. In one study, an attempt was made to investigate islet differentiation using mouse embryonic stem (ES) cell methodology. When appropriate cell lines were maintained in vitro, they appeared to be capable of differentiating as multipotent precursor cells.[108] Some lines demonstrated that they were capable of differentiating into early endocrine cell progenitors and even into islet hormone-producing cells. When incubated in nonselective medium, some were capable of spontaneously differentiating into cells that expressed each of the four major islet hormones: insulin, glucagon, somatostatin, and pancreatic polypeptide. The first cells to differentiate were cells immunostained for the pancreatic polypeptide, and only later did the hormone-positive cells appear. The possibility exists that the methodology employed here will one day be

capable of being adapted to allow the development and culture of *human* ES cell lines that will eventually hold out the possibility of transplantation to islet-deficient patients.

The transplantation of human pancreatic islet β cells clearly represents a potential means of treating type 1 (insulin dependent) diabetes mellitus. The principal technical difficulty to date is that the β cells available for grafting are extremely scarce, and new sources would have to be found to allow this sort of therapy to be undertaken. It has recently been suggested that undifferentiated islet precursor cells could provide such a source. To date, attempts have been made to graft human embryonic pancreata that contain relatively few β cells into β cell deficient, severe combine immunodeficient (*Prkdc*^{scid}/*Prkdc*^{scid}) mutant mice.[109] In preliminary experiments, the human pancreatic tissue increased in weight by a factor of about 200 times during the 6-month period of the experiment. More importantly, the number of β cells increased by a factor of closer to 5,000. The graft was mature enough to control the hyperglycemia of the β cell deficient severe combined immunodeficiency mice. It was suggested that this approach might provide a source of immature cells that could proliferate and differentiate into large numbers of β cells following transplantation. At the present time, however, this approach is still very much at the experimental stage and is capable only of providing a means of studying the development of the human pancreas during the prenatal period.

When a small component of the human fetal pancreas that consisted of β cells was transplanted to human recipients, it was shown to be capable of normalizing the blood glucose levels of diabetic patients. This effect, however, can take several months to achieve, during which time a proportion of the cells within the graft tissue proliferate and differentiate into β cells.[110] In a preliminary study, Si et al.[110] transplanted human fetal pancreas under the kidney capsule of immunodeficient mice, and the grafts were analyzed 3 months later using antibodies against its exocrine and endocrine components. While the graft's exocrine components eventually decreased, the endocrine components increased from an initial value of about 7% to about 11% of the graft after about 2 months. It is believed that after transplantation of the pancreatic tissue, exocrine cells mostly differentiate into duct cells and that these eventually develop into endocrine cells, particularly β cells.

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7 The Pineal Gland

INTRODUCTION

As most readers of this volume are likely to have only the most superficial knowledge of the development, histological morphology, and endocrine functions of the pineal organ, it was considered appropriate to discuss these aspects in slightly more detail in this chapter than might otherwise have been the case. Similarly, as the clinical management of tumors of this region, although relatively rarely encountered, are becoming of increasing importance, particularly in neurosurgical practice, it is likely that in the relatively near future strains of mice will be produced that have a predisposition toward tumors of this region. As the histological features of these tumors are so diverse, it is important that a great deal more needs to be learned about their early development and the factors that influence the early development of this gland than is presently known (http://www.emedicine.com/med/topic2911.htm).

It should be noted that an extensive magnetic resonance imaging (MRI) study has recently been carried out to determine the size of the normal pineal gland in infants, children, and adolescents.[1] While no difference was observed in the overall size of this gland between males and females, considerable variation was noted in its size among all age groups studied. It was suggested that this study, by providing baseline information on pineal size in different age groups, might be important in the detection of neoplasms of this gland.

A considerable amount of information has been published on the prenatal development of the pineal gland, also termed the *epiphysis cerebri*, in both the rat and in the human, although relatively little has been published on the morphology of this gland in the mouse. The information presently available is largely based on the anatomical, histological, and ultrastructural features in a number of mammalian species, including those indicated above, and is considered here.

When fully formed, the pineal gland is reddish-gray in color and, in the human, is about 8 mm in length. Its base is directed backward and is attached to the roof of the diencephalon by a peduncle. Its morphology in the human has been numerously reviewed.[2–4] The pineal gland retains its light sensitivity in lower vertebrates, such as in fish, amphibians, and reptiles, and has evolved as a strictly secretory organ in the higher vertebrates.[4–7] The pineal is the source of *melatonin* in mammals.[5] The complex neural pathways from the retina provide an indirect and rather complex means by which light regulates the secretion of melatonin.[8]

The pineal gland is said to consist of *primordial* photoreceptive cells,[8] and that light-encoded information is relayed to the pineal in an indirect and multisynaptic fashion.[9] Circadian rhythms thus allow adaptation to the external environment and in mammals are regulated by special "circadian timing systems." The primary pathway from the visual photoreceptors is via the retinohypothalamic tract, and these fibers either pass through or terminate in the suprachiasmatic nuclei of the hypothalamus or the superior cervical ganglia. The output from them is principally to the hypothalamus, the thalamus, and to the basal part of the forebrain. The information that passes along this route therefore influences many physiological, endocrine, and psychomotor functions concerning the sleep–wake cycle. *Melatonin* released from the pineal therefore plays a critical role in regulating such cycles. Noradrenergic sympathetic nerve terminals innervate the gland and regulate melatonin production and release.

In an early study, when there was in fact some doubt expressed as to whether the pineal gland was an endocrine organ, it was its secretion of melatonin and the influence of this substance on various endocrine organs that convinced the authors of its endocrine status.[10] This substance had

previously been shown to have an effect on the rat and hamster ovary[11,12] and hamster testis[13] and on the secretion of luteinizing hormone (LH) from the pituitary gland.[14] It was also known at that time that the pineal gland had an influence on the timing of onset of puberty in man and in animals, while pinealectomy could advance the onset of puberty in animals.[15] This study confirmed that melatonin had a positive influence on testicular development.[10]

EMBRYOLOGICAL FEATURES IN MAMMALS

In all mammalian species studied, the pineal gland arises in the dorsal midline from the caudal part of the roof plate of the third ventricle of the brain. It develops from an evagination of the most caudal part of the prosencephalon, just rostral to the cranial limit of the mesencephalic flexure. At this relatively early stage of development, therefore, the forebrain contains a central cavity termed the third ventricle, and this corresponds to the lumen of the prosencephalon. Very shortly afterward, the prosencephalic vesicle develops a number of evaginations. The earliest of these to appear are the paired optic stalks and, ventrally in the midline, the pituitary stalk or *hypophysis cerebri*. The two laterally located telencephalic hemispheres, each with its associated vesicles, appear shortly after this time. Dorsally, the first evidence of a pineal recess is also seen at about this time. Because the pineal gland projects caudally, it lies immediately dorsal to the roof of the midbrain (the mesencephalic tectum).

Shortly thereafter, two sulci develop within the lateral or diencephalic walls of the third ventricle. These are the hypothalamic and epithalamic sulci. The former corresponds with the upper extent of the hypothalamus, while the latter divides the rest of the diencephalon into a thalamic region ventrally and the epithalamic region dorsally. As development progresses, the thalamus grows considerably more rapidly than the epithalamus so that, with time, the latter region occupies a relatively small part of the most dorsal region of the diencephalon. The pineal gland forms as one of the principal parts of the epithalamus. The latter also includes the habenular commissures and the trigonum habenulae. These develop in association with the caudal part of the roof plate and the adjoining regions of the lateral walls of the diencephalon. The habenular nuclei form within the mantle zone of the epithalamic region, adjacent to the pineal stalk. Fibers pass caudally from the habenular nuclei into the peduncular region of the mesencephalon (the so-called *habenulopeduncular tract*). Two transversely running commissures also develop: one dorsal (or cranial) to the pineal gland (the *habenular commissure*) and the other ventral (or caudal) to the pineal gland (the *posterior commissure*).

The region of the roof plate of the original prosencephalic vesicle becomes extremely thin, and part of it gives rise to the choroid plexus of the third ventricle (the so-called *tela choroidea* of the third ventricle). This is morphologically similar to how the choroid plexus forms in the roof of the fourth ventricle of the brain. Other outgrowths that develop in close proximity to the pineal gland, although unrelated to it, also form in the roof plate of the third ventricle in the human. Although these usually completely regress, they may occasionally persist, often giving rise to thin-walled cysts. One of these is termed the *paraphysis*.[16] If this structure persists, it may give rise to clinical sequelae.[17]

During the early part of its differentiation, the proximal part of the pineal primordium, or stalk, remains hollow and is entered via the pineal recess of the third ventricle. The gland itself is located directly posteriorly or caudally to the stalk. Its distal part remains hollow for a considerable time, but eventually becomes solid due to cellular proliferation within its walls. Its neuroglia differentiates into the pineal cells, while, during the postnatal period, nerve fibers pass from the epithalamus mostly, although not exclusively, along the pineal stalk. The apex of the pineal gland is closely related to the great cerebral vein.

In many reptiles, the pineal outgrowth is initially a double structure with anterior and posterior components. The anterior part (termed the *parapineal organ*) forms the pineal or parietal "eye," while the posterior part has a glandular appearance. In the human embryo, the anterior part develops to a limited degree but usually regresses completely. Ultrastructural evidence supports the

hypothesis (see below) that the pineal gland in the human is a homolog of the third or parietal eye of the lower vertebrates, although this is not a universally accepted view.[18]

PRENATAL DEVELOPMENT OF THE PINEAL GLAND IN RODENTS

Despite an extensive literature search, virtually all the studies on the *development* of this gland in rodents so far located appear to have been undertaken in the rat. Apart from a few descriptive studies carried out in the mouse, the findings from the rat are discussed here first; then the descriptive information from histological studies carried out in staged mouse embryos at comparable prenatal periods are reviewed. It should also be noted here that, with only a few exceptions, all the articles in which *mice* were studied involved the analysis of *postnatal* rather than *prenatal* stages of development. Furthermore, the majority of these studies were undertaken to determine the effect of altering the light–dark cycle on, for example, melatonin release and to investigate its quantitative effect on the ultrastructural components of the gland. These observations are then followed by the descriptive analyses of the developmental features observed in other mammalian species, such as the sheep and human, where comparable information is available.

Clabough[19] appears to have carried out one of the first studies in which a detailed histological analysis was undertaken to investigate the development of the pineal gland in both the rat and hamster. This was followed up by a number of studies by Calvo and Boya[20] in which they reported their histological and ultrastructural findings on the development of this gland during the late prenatal period. In a number of subsequent studies, they reported on the cellular morphology of this gland during the postnatal period and in the adult. In Clabough's study, distinct similarities were observed between the development of the pineal gland in the rat and hamster. In the rat, for example, it was noted that the development of this organ occurred during the last 8 days of gestation (days 14–22), while in the hamster, it occurred during the last 5 days of gestation (days 11–16). It was noted that by day 16 of gestation in the rat the pineal was first evident as an elongated simple tubular outpocket from the roof of the diencephalon and that its wall was five or six cell layers thick. It was also noted that the tela choroidea was slightly folded and projected into the third ventricle. The pineal cells stained only to a limited extent and possessed a large basophilic nucleus. The apical borders of the cells that lined the lumen of the gland primordium also possessed rounded projections. These findings were similar to those described previously by others.[21]

There were many other similarities noted between the pineal gland in these two species. For example, as differentiation proceeded, it was noted that the lumen of the gland tended to disappear. This was also evident with regard to the histological morphology of the cells that bordered the lumen. Many of these cells contained cilium-like structures and "ellipsoid-like" apical cytoplasmic bulges that often contained one or two centrioles and a ciliary-like derivative. It was suggested that this was evidence for the hypothesis that the cells in the mammalian gland were probably derived from the photoreceptor cells characteristically seen in the lower vertebrates.[22] Others had previously noted the occasional presence of these ellipsoid-like processes in some persisting follicles in adult rat pineal glands.[23] By day 18 of gestation, the pineal cells were more closely packed than previously and tended to be randomly orientated, while the cells that bordered the lumen contained cytoplasmic protuberances. These cells also contained numerous mitotic figures. By day 20 of gestation, the pineal lumen had largely disappeared distally but was still evident proximally. In the gland itself, the lumen had invariably disappeared, while the cells often appeared to be arranged as rosettes. Numerous venous channels and capillaries were now evident within the body of the gland. The appearance at full term (day 22) was very similar to that seen in the day 20 fetuses. Few, if any, follicles were now evident, and the organ consisted of randomly arranged cells and a small number of rosettes.

At the ultrastructural level, the intracellular features observed previously were now confirmed. It was also possible to see in greater detail the surface morphology of the circumluminal cells. One or two centrioles were often observed within the cytoplasmic bulges that extended into the pineal lumen. Cilium-like processes were also occasionally noted that extended for a short distance

into the pineal lumen. No obvious difference was observed in the cytoplasmic features of the 20- compared to the 18-day glands. Similarly, no neural elements were observed in any of the fetal organs studied. There was also no evidence in these prenatal preparations of the distinct cell types described in the adult pineal gland (see below). The only ultrastructural feature of interest noted was the presence of the apical cytoplasmic bulges that often bore cilium-like processes, and these were described as being similar in morphology to the developing photoreceptor cells seen in certain submammalian vertebrates.

In their initial descriptive study on the features of pineal gland development from day 13 of gestation until birth in the rat, Calvo and Boya[20] noted that the first evidence of its differentiation was as a small midline evagination in the roof of the diencephalon. Shortly afterward, the pineal primordium was seen to have a tubular morphology. By about 17 days of gestation, the pineal recess had almost completely disappeared, while the gland was transformed into a more solid structure. This was principally due to infolding and thickening of its dorsal wall. It was also evident that most of the parenchyma of the future gland appeared to have been derived from this region of the gland. Similarly, most of the blood vessels observed in the gland appeared to have been derived by their invasion into its dorsal surface.

In a complementary study, in which their ultrastructural findings were reported, Calvo and Boya[24] noted that little evidence of epithelial differentiation was observed before about 17 days of gestation. After this time, two mechanisms appeared to be involved in the obliteration of the pineal recess. This was initially through multiple folding of the epithelium, which produced a considerable degree of approximation and fusion of its walls, and secondly through the filling of the lumen by cells that appeared to have been extruded from the pineal epithelium. Despite the fact that the pineal recess was almost obliterated by these mechanisms, some evidence of its presence was still seen at the time of birth (at about 21–22 days of gestation). It was also noted that from about 16.5 days of gestation until birth, increasing evidence of cellular differentiation was observed within the pineal gland, although by full term Calvo and Boya were unable to distinguish between the various cell types present with any degree of certainty. Such cells at this immature stage of their development are therefore referred to as *pinealoblasts*. Their principal feature was the presence of scanty cytoplasm and frequent mitotic figures.

ULTRASTRUCTURAL MORPHOLOGY OF CELL TYPES IN THE POSTNATAL RAT PINEAL GLAND

In a follow-up study by Calvo and Boya, the cellular morphology of the pineal gland in the early postnatal rat was studied. This analysis was complemented by a study of the cellular features of the pineal gland in pubertal and adult rats.[25] The authors noted that present in this gland were two distinct cell types of parenchymal cells, or pinealocytes, that were readily distinguished by their ultrastructural features. Others had described such cells,[23,26] although they had been given different names by different authors.[27] The time of onset of differentiation of these two cell types has variously been described in the rat. Some have indicated that two cell types are first recognized at between 10 and 14 days after birth and that all the cell types present in the neonatal rat were undifferentiated.[25,28] Others claimed to have recognized these two cell types by as early as 2 days after birth from cells established in tissue culture.[29] The principal aim of Calvo and Boya's study was to establish the time scale of development of these and the other cell types recognized in the pineal gland of the rat during the postnatal period. Pineal preparations were therefore processed at intervals between 1 and 60 days after birth, and the ultrastructural morphology of these glands was carefully examined.

Early evidence of pineal cell differentiation was first seen in this study on postnatal day 3. It was possible to recognize both Type I and Type II pinealocytes at this time (for their cellular features, see below). Nerve fibers were also recognized in the connective tissue spaces of the gland and frequently appeared to be in contact with the Type II pinealocytes. By postnatal day 10,

the number of pinealoblasts present decreased progressively, and these cells were now replaced by differentiating pinealocytes. Although these cells possessed only a limited amount of cytoplasm, they were readily recognized at this time by their characteristic features. By postnatal days 15–20, the parenchyma of the gland had a typical cord-like appearance. Both cell types were by now readily recognized, and their morphology was clearly different from the cells seen at earlier stages of development. By day 20, two varieties of Type I pinealocytes were distinguished on the basis of their different nuclear morphology. Type Ic cells possessed a clear and uniform nuclear appearance, while Type Id cells possessed a uniformly dense nuclear morphology. Despite this, both subtypes were readily distinguished from the Type II variety. The process of cellular differentiation in these cells now appeared to be complete. By postnatal days 40–45, all of the features recognized in adult pineal cells were now present. Evidence of centrioles and cilia were sometimes observed in the central cavity,[30] although these had usually disappeared by about 15–17 days postnatally. Furthermore, when a direct comparison was made between neonatal pinealocytes and developing photoreceptor cells, a considerable degree of similarity was observed. By this stage, the centrioles usually became transformed into a cluster of microtubules joined by dense material, as previously described by Lin.[31]

The features of these mature cells in pineal glands as observed in adult rats[25] are as follows:

***Type I pinealocytes*:** These cells displayed extensive cytoplasm that was rich in organelles, and this was associated with rough and smooth endoplasmic reticulum throughout the cell. In certain areas of these cells, usually close to the cell membrane, more abundant rough and smooth endoplasmic reticulum was observed than elsewhere in these cells. A Golgi complex was a prominent feature. Mitochondria were numerous and variable in form. Lipid droplets and free polyribosomes were also present in these areas. Most of these cells possessed small dense bodies that were generally found in groups that were related to vacuoles and multivesicular bodies, and such vacuoles often contained remnants of organelles. From shortly after birth, groups of junctional complexes were present between these cells, and these complexes increased progressively after about postnatal day 15.

***Type II pinealocytes*:** Rough endoplasmic reticulum was particularly commonly seen in Type II cells and most possessed one or more vacuoles, some of which contained others inside it. Complexes resembling gap junctions were usually present between these cells. Connective tissue spaces between these cells were relatively large and abundant. It was noted that any nerve fibers present were usually closely related to the Type II cells. It is believed from the tissue culture studies of Steinberg et al.[29] that in the absence of innervation, typical Type II cells do not develop. It was also noted that no junctional complexes were observed between Types I and II cells.

Early histological studies of the pineal gland in the postnatal rat[32] revealed that cell proliferation was rapid up to about 2 weeks after birth and then dropped to a lower level of activity until adulthood was achieved. The gland also increased in volume from about this time until about 10 weeks of age when a maximum size was achieved. The study by Quay and Levine also indicated that the change in size of the gland was primarily due to hypertrophy of the parenchymal cell population. The findings from this study were confirmed in the late 1960s when Altman and Das[33,34] injected tritiated thymidine into infant rats. They noted that a high proportion of the labeled cells associated with the brain were located in the endocrine glands, such as the pituitary and pineal. They further noted that the labeled cells were either neural precursors or neuroglia-like cells. Other autoradiographic studies in the late 1960s also investigated in greater detail the postnatal development of the pineal gland in this species.[35] These findings demonstrated that cellular proliferation within the gland occurred in the neonatal animal and that it continued but at a decreasing rate into adulthood. It had also been revealed that the increase in the volume of the gland in the young animal[36] was principally due to cellular hyperplasia, while in the adult it was largely due to cellular hypertrophy. These studies also

confirmed that the parenchymal cells were the principal cellular component of the gland that was involved in these processes.

In another study by Calvo and Boya,[37] the histological appearance of the rat pineal gland from birth to 10 months of age was described. This was a more detailed study than they had previously carried out.[25] By postnatal days 5–10, they now noted that the parenchymal cells began to increase in size. At the end of this period, two types of pinealocytes were recognized by their nuclear morphology, although many of the parenchymal cells still displayed the immature nuclear appearance observed in younger animals. By postnatal days 15–20, the parenchymal cells were now arranged in cords. In the periphery, groups of nuclei were radially arranged around a central space forming "pseudo-rosettes." By 25–30 days, they noted that the number of cells per unit area of the gland decreased to 35–40% of the number present during the first days after birth. Mitoses were now also rarely seen. The two cell types they had earlier described (the types I and II pinealocytes) were now readily recognized. Type I pinealocytes possessed large round nuclei with pale chromatin and prominent nucleoli and now formed 85–90% of the parenchymal cells present (also sometimes termed *pinocytes*), while type II pinealocytes possessed smaller ovoid nuclei and contained dense and homogeneous chromatin. The nuclei of endothelial and connective tissue cells were smaller and denser than were the type II cell nuclei.

In the adult rat, from about day 75 onward, more gradual changes were observed. The size of the gland increased slowly from about 75 days to 6 months of age. They also noted the presence of other cells in the pineal stroma, such as striated muscle[21,38,39] and lymphoid cells.[40] The striated muscle, while only occasionally seen, was usually located in association with the septa beneath the capsule of the gland, while the lymphoid cells were first observed at about 45 days after birth and constantly thereafter. The lymphoid cells usually infiltrated the tissue septa and the neighboring parenchymal tissue. Occasionally, large nodules of lymphoid tissue were observed near to the capsule of the gland and extended from there into the interior of the gland.

Previously, Tapp and Blumfield[39] had described the differentiation of the pineal cell types and had recognized three types of cells (termed I, II, and III). They indicated that their Types I and II cells first appeared at about 2 weeks after birth and that their Type III cells were first recognized at about 4 weeks after birth. Calvo and Boya[37] indicated that Tapp and Blumfield's Types I and II cells were simply variants of their own Type I cells,[25] while Tapp and Blumfield's Type III cells corresponded to their own Type II cells.

In a study of glial cells present in the rat pineal gland between birth and adulthood, immunohistochemical stains were used to demonstrate the expression of a number of glial antigens, such as vimentin (VIM), glial fibrillary acidic protein (GFAP), and S-100 protein.[41] A network of VIM-immunopositive cell cords that formed a network throughout the gland were demonstrated from as early as the first few postnatal days, but these progressively disappeared during the first postnatal month. By about 20–25 days after birth, these VIM-immunopositive cells were seen only as isolated star-shaped aggregates of cells within the parenchyma of the gland. A similar developmental pattern was observed with the other glial-specific proteins, although both of these were first noted only at about 15–20 days after birth. At all times they were located in the proximal one third of the gland and were either within or very close to the pineal stalk.

In a subsequent study by members of the same group, the effect of chemical denervation of the glial cells in the rat pineal gland was assessed during the postnatal period.[42] Denervation was assessed by the immunohistochemical expression of the three antigens characteristic of pineal glial cells indicated previously. Denervation was carried out either by administration of an appropriate neurotoxic agent during the first 5 days after birth or by injecting pregnant rats with another neurotoxic agent on day 15 of gestation. VIM immunoreactivity was detected from the first postnatal day both in denervated and control animals. In the denervated glands, glial cell maturation was considerably accelerated. After day 30, the number of these cells increased until adulthood when the denervated gland had a reticular appearance. This was due to the presence of large numbers of immunoreactive cell processes. The other glial proteins appeared earlier in the

denervated glands than in the controls, and their number and size was considerably greater at all stages compared to those seen in the controls. These cells were characteristically present in the proximal half of the gland.

OTHER STUDIES UNDERTAKEN ON THE PINEAL GLAND IN THE RAT

Curiously, it was demonstrated that the pineal gland in the rat contains high levels of rhodopsin kinase, previously thought to be an exclusively retinal enzyme.[43] Furthermore, this enzyme was present some days earlier in the pineal gland than in the retina. A 25% increase in the level of this enzyme was observed in the middle of the dark and beginning of the light period, while exposure to constant light caused a 50% decrease in its level in both tissues. Only the pineal enzyme activity declined following bilateral superior cervical sympathectomy, indicating that it was controlled by light activity via the sympathetic nervous system. This is consistent with a role of this enzyme in the neural regulation of pineal function.

In another study, the effect of photoperiod on testicular development in prepubertal rice rats demonstrated that the duration of the photoperiod in this species was of critical importance.[44] When male marsh rice rats were gestated and reared during the first 28 days after birth in photoperiods ranging from 8 to 13 hours of light per day, their testicular development was *inhibited*. When reared in 14 or more hours of light per day, testicular growth was *stimulated*. The critical photoperiod was found to be between 13 and 14 hours per day for stimulating testicular growth. Exposure to these photoperiods during the period of lactation, during days 1–14 after birth, particularly affected testicular development and was the primary factor determining whether testicular maturation would occur. Pinealectomy, by contrast, had minimal influence on the magnitude of the effect on testicular development when animals were maintained on the presurgical photoperiod but prevented animals from demonstrating an appropriate response after surgery.

A study[45] examining the influence of the maternal pineal gland on ovarian cycle development in prepubertal female offspring also complemented the other work.[44] There it was suggested that the maternal pineal gland could influence fetal development because melatonin crosses the placental barrier. Treatment of pregnant females throughout gestation with melatonin injections delayed sexual maturation in the female offspring. Another group of females was pinealectomized during pregnancy. The ovaries of the female offspring from both groups were removed and examined histologically at 25, 30, or at 34 days after birth. In the melatonin-treated females, ovarian development in their female offspring was detrimentally affected at each of the time points studied. In the female offspring of the pinealectomized mice, no significant difference in oocyte cellular volumes was observed, although a significantly higher nucleolar volume was seen at 25 days and a lower nucleolar volume at 30 days. This study indicated that the maternal pineal gland participates in prepubertal oocyte development, being particularly evident after melatonin treatment during pregnancy.

Earlier studies had shown that melatonin influences seasonal breeding in sheep and hamsters, and another demonstrated a similar effect in the rat.[46] Both male and female rats are sensitive to daily injections of melatonin at the beginning of sexual maturation. The male is most sensitive between days 20 and 30 after birth, with a delayed onset of sexual development occurring after 45 days whether the injections are discontinued or maintained. In female rats, daily injections of melatonin during the prepubertal period delays vaginal opening and disrupts the first few oestrus cycles. Melatonin is particularly effective in this regard, especially if given in the late photoperiod. It was suggested that melatonin interfered with the control of the secretion of gonadotropin-releasing hormone and that, in addition to influencing seasonal breeding, it may play an important role in the timing of onset of sexual maturation.

In one of the most recent papers published involving this species, the bromodeoxyuridine labeling method was utilized to evaluate the proportion of cells within the pineal gland that were in the S-phase of the cell cycle.[47] Representative paraffin sections of the pineal gland from 1-, 7-, 14-, and 28-day-old rats were studied. The proportion of cells in the S-phase during the first week after birth

decreased from about 9% to 1.3%. A considerably smaller decrease was observed between the 7th to the 14th postnatal day, when the incidence was then less than 0.5% of the total cells examined. Between the 14th to the 28th days, an even lower proportion of the cells were in the S-phase. The total number of cells per unit area of the pineal gland in these sections decreased sharply from birth until the end of the first week and then less sharply until the end of the first month. It was suggested that these findings indicated that an increase in cell population preceded morphological and functional maturation within the rat pineal gland.

EXPOSURE OF PRE- AND EARLY POSTIMPLANTATION STAGES OF MOUSE DEVELOPMENT TO PINEAL INDOLES

Chan and Ng[48] carried out the only known study to date on preimplantation mouse embryos. Mouse embryos were exposed at either 1.5 or 2.5 days *post coitum* (dpc) to a range of pineal indoles, such as hydroxyindoleacetic acid (HIAA), melatonin, methoxytryptophol (MTP), or methoxytryptamine (MTA), both in vivo and in vitro. Appropriate controls were also studied, and all mice from the in vivo studies were sacrificed at 17.5 dpc. No obvious detrimental effects were induced in the majority of cases, although some mice treated with MTP or MTA produced litters in which all of the embryos were resorbed. Four-cell stage embryos that were exposed in vitro were examined after 24, 48, 72, or 96 hours. It was noted that MTA retarded embryonic development at all time points studied, while HIAA also produced a slight inhibitory effect on embryonic development. Some of the embryos exposed to MTA and HIAA underwent degeneration. Exposure to melatonin and MTP usually had no detrimental effects. When exposure occurred at the eight-cell stage, MTA induced only a minimal retarding effect, while the other indoles had no obvious detrimental effect on the development of these embryos. It is, of course, unclear whether the doses of these indoles bore any relation to those that would normally be present in the reproductive tract in vivo.

In a complementary study,[49] Chan and Ng gave subcutaneous injections of these pineal indoles (1 mg/25 g body weight) at 8.5 dpc and noted that this dose of these agents produced no adverse effect on embryonic development. However, exposure in vitro to various doses of melatonin, MTP, and MTA at the early somite stage of development induced an increase in the incidence of congenital abnormalities of all of the systems analyzed when this was assessed at 10.5 dpc.

DEVELOPMENT OF THE PINEAL GLAND IN STAGED MOUSE EMBRYOS

Figures 7.1 through 7.8 illustrate the development of the mouse pineal gland. All of the embryos in this section have been carefully staged according to the Theiler staging scheme and are referred to as being at various Theiler Stages (TS).[50,51] The first evidence of a pineal primordium is clearly seen in the mouse embryo at TS 21, equivalent to 13.5 dpc, when a fairly deep evagination is seen in the roof of the diencephalon. Embryos were therefore examined at approximately 24-hour intervals between this stage and full term, equivalent to TS 26 (17.5 dpc). The following stages were therefore studied: TS 21 (13.5 dpc); TS 22–23 (14.5 dpc); TS 24 (15.5 dpc); TS 25 (16.5 dpc); and TS 26 (17.5 dpc). According to this staging system, TS 26 is said to be the degree of development achieved at full term in this species. All of the embryos illustrated here were sectioned either in the transverse, sagittal, or coronal plane, and a number of these are from embryos previously illustrated in *Atlas of Mouse Development*.[52] All of these histological sections were stained with hematoxylin and eosin (H&E) and cut at a nominal thickness of between 5 and 7–8 µm, depending on the stage of development. No evidence of a paraphyseal or dorsal diverticulum was seen in any of the sagittal sections through this region of the roof of the diencephalon:

TS 21 (13.5 dpc): In median sagittal sections cut through the midline region of the roof of the diencephalon, an obvious tubular evagination is observed in this region. The impression

is gained that the neck of the gland is wide, while the gland itself is not particularly deep. Much of its wall possesses about four to six layers of cells (Figure 7.1, Figure 7.2). This represents the first evidence of the pineal primordium, although no evidence of projections of the tela choroidea into the third ventricle were seen at this stage. There was also, therefore, no evidence of choroid plexus material evident in this region other than the relatively small amount that projected into the lateral ventricles. The appearance was very similar to that observed in the rat at about 15.5 dpc and in the chick embryo on the fourth day of development. Little is seen on transverse sections through the pineal primordium at this stage.

TS 22–23 (14.5 dpc): In sagittal sections through the gland at this stage, the opening in the region of the pineal recess is slightly narrower, while the tubular body appears to be directed more obviously caudally than previously. The wall of the gland now appears to contain about 8–10 layers of cells.

TS 24 (15.5 dpc): In sagittal sections of the gland at this stage, while the pineal recess remains wide, the distal part of the gland appears to be far more solid than previously and slightly more caudally directed. Considerable evidence of choroid evaginations is now seen some distance rostral to the pineal recess (Figure 7.3, Figure 7.4). Coronal sections through the pineal recess and gland at this stage are particularly informative in that they emphasize the transversely running posterior commissural fibers located between the recess and the body of the gland. These commissural fibers are also clearly seen in the transverse sections through the gland at this stage. While the entrance to the pineal recess in the coronal sections is still clearly seen, the lumen of its stalk appears to be filled with cells. The body of the gland now appears to consist of a considerable number of rosettes, while its dorsal surface is indented by a large diameter blood vessel, possibly the superior sagittal dural venous sinus or the great cerebral vein.

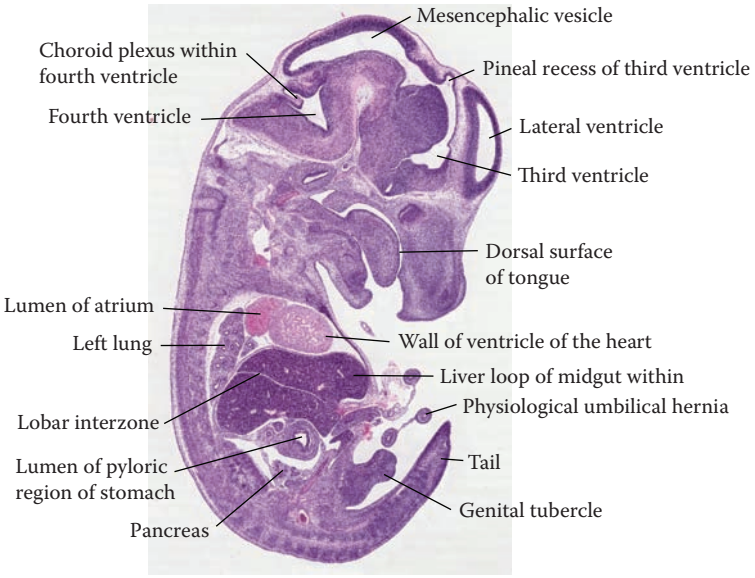

FIGURE 7.1 (E12.5_Fig7.1.svs) Low magnification, sagittal section just to the left of the median plane in the abdominal region but close to the median plane in the upper anterior part of the cephalic region of a mouse embryo (E12.5–13.5). The section is stained with H&E.

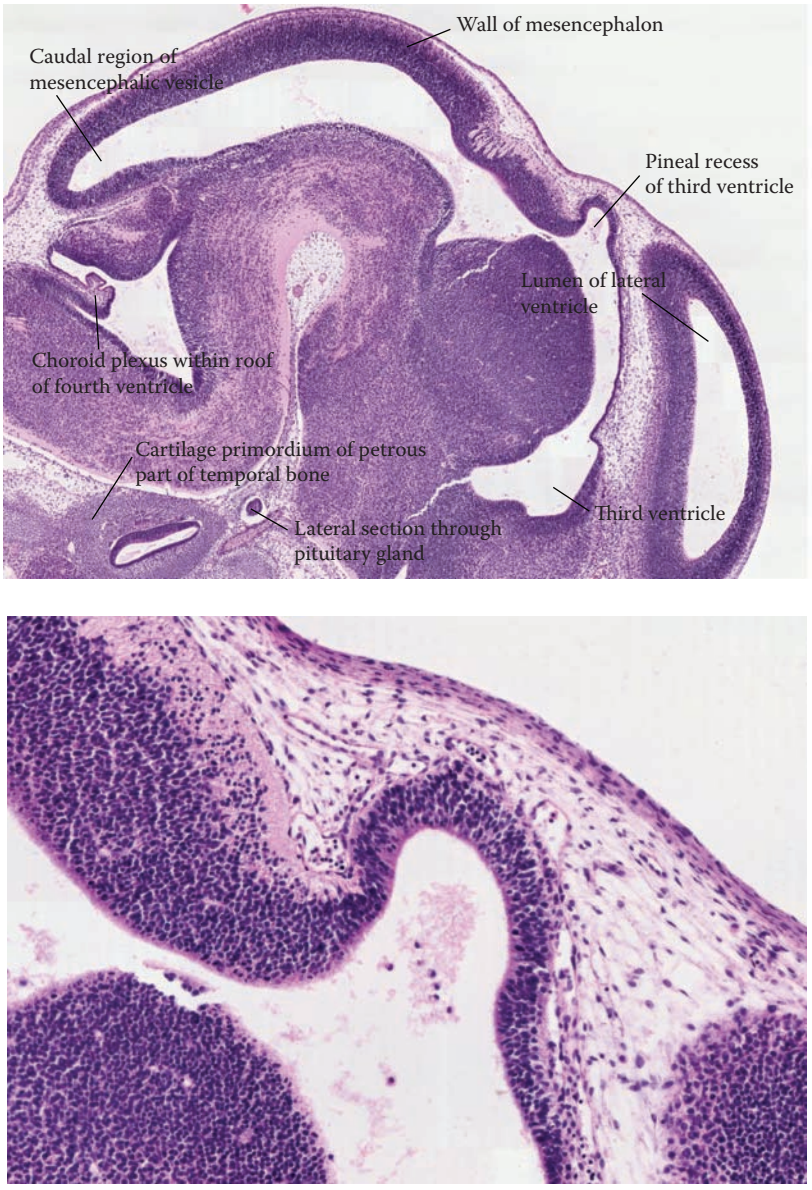

FIGURE 7.2 Medium (top) and high (bottom) magnification of Figure 7.1 illustrates the rostral part of the third ventricle, close to the central part of the roof of its pineal recess. This represents a very early stage in the development of the pineal gland. The region through the pituitary gland indicates that this section is some distance from the median plane in this area.

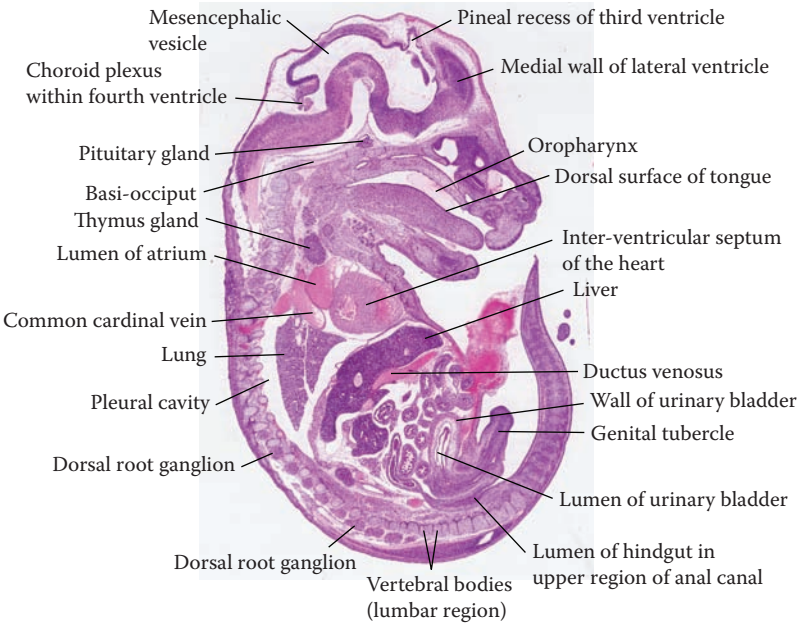

FIGURE 7.3 (E14.5_Fig7.3.svs) Low magnification, sagittal section just to the right of the median plane in the abdominal region and close to the median plane in the upper part of the cephalic region of the mouse embryo (E14.5–15). This section is stained with H&E.

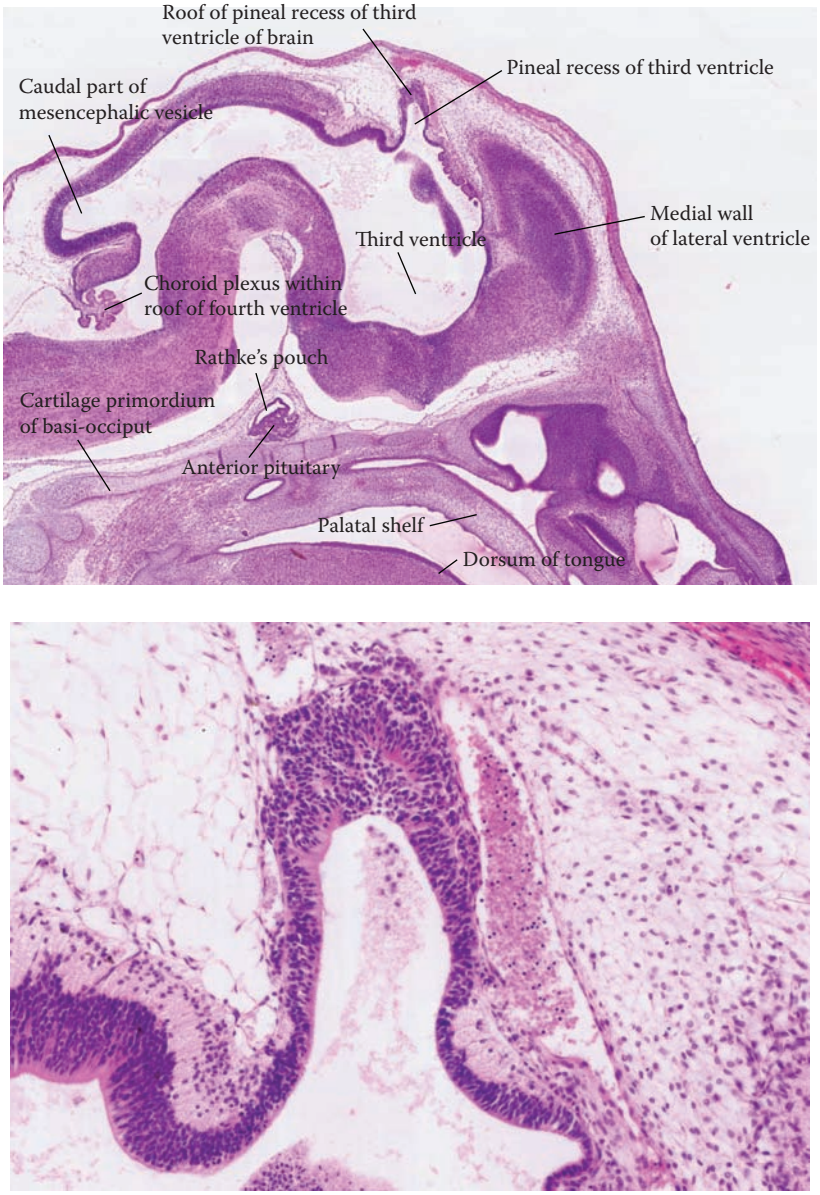

FIGURE 7.4 Medium (top) and high (bottom) magnification of Figure 7.3 shows the rostral part of the third ventricle, close to the central part of the roof of the pineal recess. The roof of the pineal recess shows evidence of thickening, although its connection with the third ventricle of the brain remains wide. No evidence of the posterior pituitary is seen in this section, indicating that it is just lateral to the median plane in this region.

TS 25 (16.5 dpc): In sagittal sections of the gland at this stage, while the pineal recess remains tubular, the lumen of the more distal part of the gland has now virtually completely disappeared (Figure 7.5, Figure 7.6). The choroid evaginations have now extended caudally so that they appear to extend almost into the entrance to the pineal recess. Transverse sections through the pineal recess indicate that its lumen is considerably narrower than previously and that a wide anuclear zone lines it. The transversely running posterior commissural fibers are also clearly seen at this stage and appear to be located between the region of the pineal recess and the body of the gland.

TS 26 (17.5 dpc): Sections through the gland indicate that the choroid plexus material now appears to extend caudally almost into the pineal recess. It is also possible to see that the body of the gland actually possesses a very narrow transversely directed lumen. This appears to be lined by a wide anuclear zone, whereas the rest of the body now appears to possess at least 10 or more layers of cells (Figure 7.7, Figure 7.8).

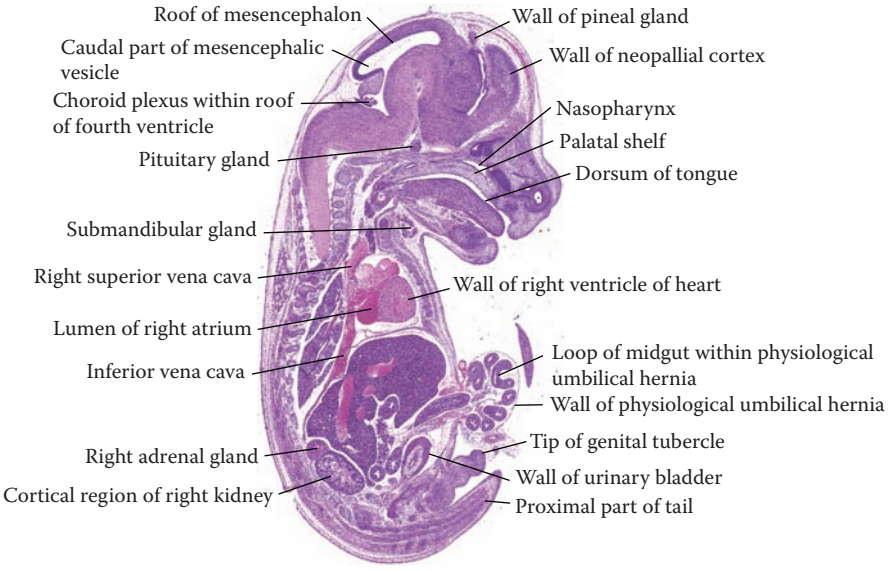

FIGURE 7.5 (E15.5_Fig7.5.svs) Low magnification, sagittal section just to the right of the median plan in the abdominal region and just lateral to the median plane in the region of the pituitary gland. This section is from a mouse embryo (E15.5–16) stained with H&E.

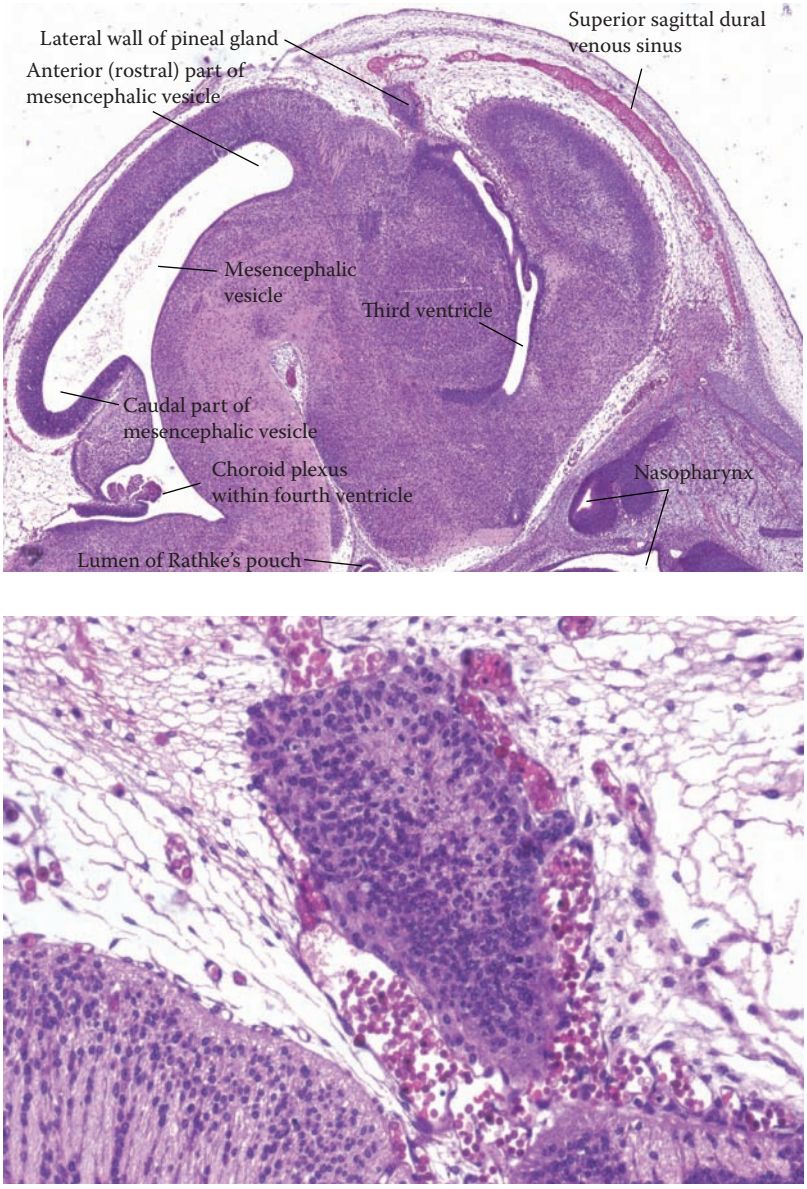

FIGURE 7.6 Medium (top) and high (bottom) magnification of Figure 7.5 demonstrates the wall of the pineal gland. The view of the pituitary gland indicates that this section is just lateral to the median plane, due to the absence of visual evidence of the primordium of the posterior pituitary gland.

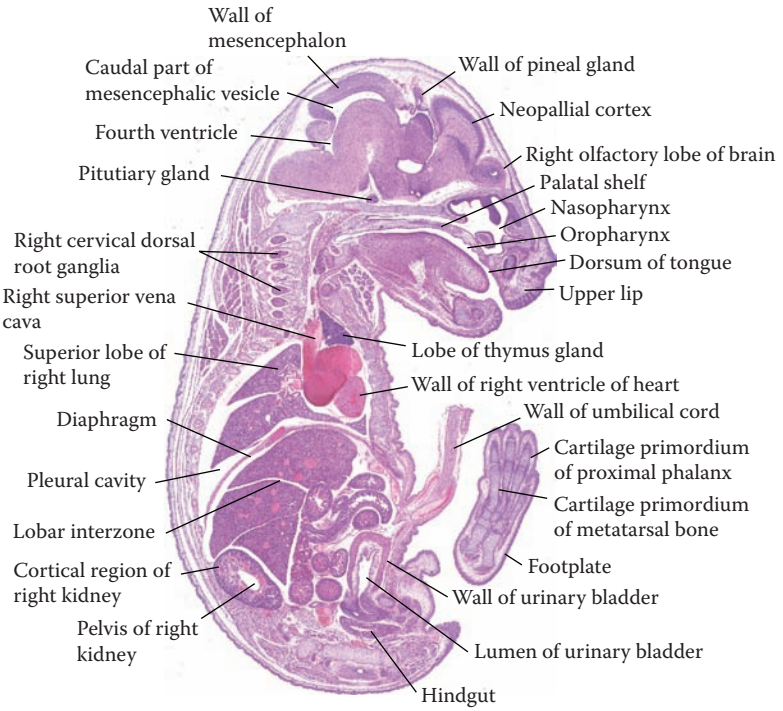

FIGURE 7.7 (E17.5_Fig7.7.svs) Low magnification, sagittal section just to the right of the median plane in the abdominal region and just lateral to the median plane in the region of the pituitary gland. This section is from a mouse embryo (E17.5–18) stained with H&E. In the abdominal region, the pelvic region of the right kidney and the lumen of the urinary bladder are clearly seen.

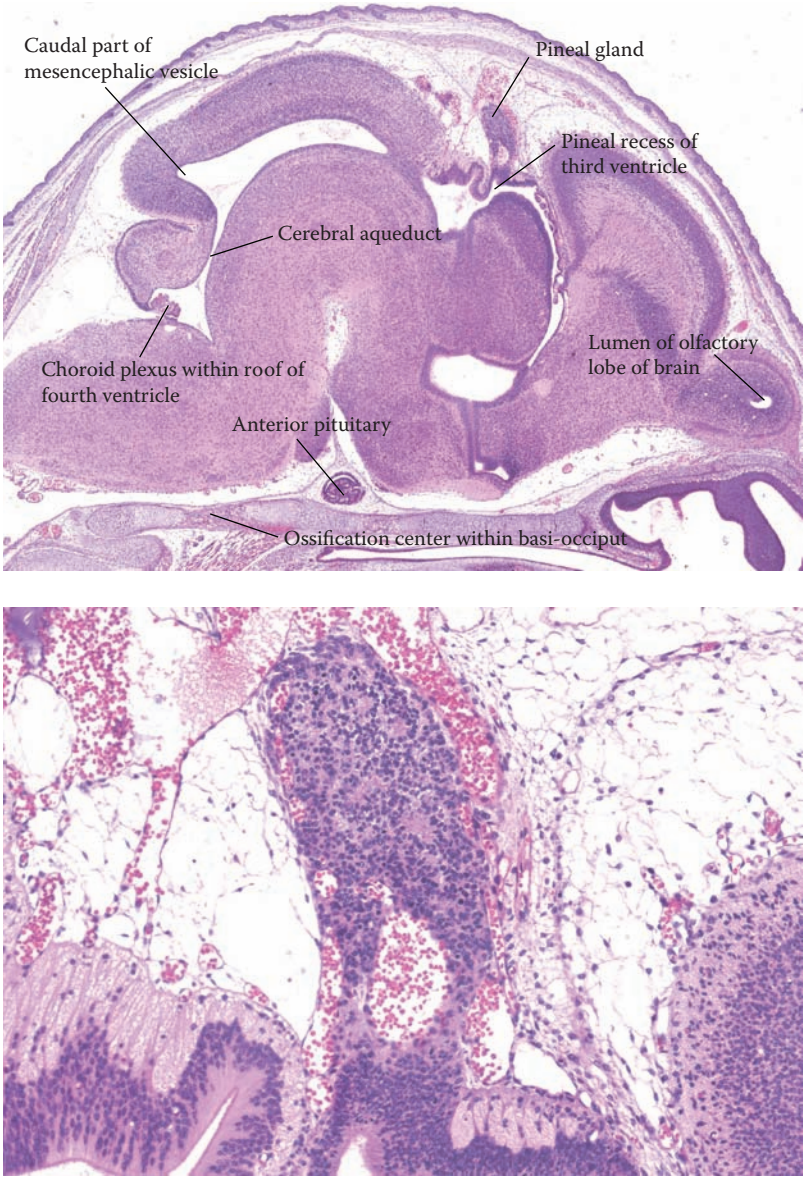

FIGURE 7.8 Medium (top) and high (bottom) magnification of Figure 7.7 shows the thickened roof of an early stage in the development of the pineal gland. The view of the pituitary gland indicates that this section is just lateral to the median plane, due to the absence of visual evidence of the primordium of the posterior pituitary gland.

STUDIES THAT HAVE BEEN CARRIED OUT WITH POSTNATAL MICE

The relatively small selection of topics presented here is fairly representative of the range of pineal studies in which mice have been used. Not surprisingly, these appear mostly to have been concerned with investigating the factors that influence the diurnal secretion of melatonin. For example, long-term monitoring of melatonin secretion was employed in a study involving the CBA inbred mouse strain that ran for over 20 days.[53] They implanted a dialysis probe either into or near to the pineal gland in these mice and noted that melatonin secretion was exactly matched by the animal's circadian change in locomotor activity. Two peaks of daily secretion of melatonin were observed. The first (small) peak occurred at the day/night transition time, while the second (larger) peak occurred at midnight. The use of various "blocking" agents suggested that in these mice melatonin release at night is activated by adrenergic signaling from the superior cervical ganglion but that the enhanced melatonin secretion observed during the daytime is not mediated by neuronal signaling.

A relatively recent study investigated the influence of continuous light and continuous darkness on the number of large granular vesicles observed in the sympathetic nerve endings located in the pineal parenchyma. Similar studies have also been undertaken to investigate whether the pinealocytes displayed a 24-hour rhythm by studying the number of the various intracellular organelles present within them at different times of the day and night.[54] The morphological appearance of the pericapillary and intercellular spaces and the capillary endothelial cells were also examined. The nuclear and cytoplasmic areas of the pinealocytes increased during the light period, whereas the area of condensed chromatin in the pinealocytes decreased during the light period. The extent of the pericapillary and intercellular spaces increased during the light period and decreased during the dark period. Diurnal variations were also seen in the cross-sectional areas of the endothelial cells. These decreased during the light period, as did the number of fenestrae. The reverse occurred during the dark period. It was suggested that these changes were related to the synthetic activity of the pinealocytes.

Earlier studies had attempted to investigate the location of the numerous sympathetic nerve endings seen in this gland and their possible relationship to the pinealocytes. A number of semiquantitative ultrastructural studies have been undertaken to investigate the diurnal variation in the number of small vesicles located in the sympathetic nerve endings observed in this gland. One study[55] examined the effect of continuous darkness on the diurnal variation in the number of these small vesicles. Mukai and Matsushima demonstrated that an influence was produced on the numerical incidence of Type I granulated vesicles (predominantly small-granulated vesicles). Nongranulated elliptical vesicles normally seen during the first half of the dark period under diurnal lighting conditions may persist for at least 14 days under periods of continuous darkness. They also noted that if mice were kept under continuous darkness for 7 days, the normal pattern of vesicles present in the Type I pinealocytes might be lost. Furthermore, they noted the presence of two types of granulated vesicles in these cells: (1) the incidence of the Type I granulated vesicles may be abolished during continuous darkness; and (2) the Type II granulated vesicles may be maintained under these conditions. It was suggested that the diurnal rhythm in the number of small-granulated vesicles in sympathetic nerve endings might be controlled by two different mechanisms: exogenous and endogenous.

In an earlier study by Matsushima et al.,[56] an attempt was made to establish the relationship between the sympathetic nerve endings and the pinealocytes. They indicated that the diurnal change in the number of small-granulated vesicles was similar to the noradrenaline content of rat pineals. An increase was seen at night, reaching maximum levels at the beginning of the light period. It was suggested that the decrease in small-granulated vesicles might be correlated with the release of noradrenaline from the nerve fibers. It was furthermore suggested that the diurnal variation in the glycogen content of the mouse pineals might also be related. They argued that light suppressed the release of noradrenaline from the nerve fibers and thus caused an increase in the glycogen content and number of granulated vesicles in the pinealocytes.

In another ultrastructural study by this group, the effect of diurnal variation on the number of small granular and nongranular vesicles as well as the effect of continuous light on the vesicles in

sympathetic nerve endings was investigated.[57] The number of small vesicles, but not the nongranulated spherical vesicles, varied diurnally in relation to the daily photoperiod. This was seen also in relation to total vesicle density. Continuous lighting abolished the diurnal variation in small vesicles and total vesicle density. A similar study was undertaken to investigate the diurnal variation in large granular vesicles in the sympathetic nerve fibers.[58] While these vesicles were almost constant in number during the daytime, minimum numbers were seen at the middle and end of the dark period, and maximum numbers were observed at the end of the light period. In a complementary study, the effect of continuous lighting and continuous darkness on the incidence of these large granulated vesicles was also studied.[59] Diurnal variation of these large granulated vesicles appeared to be controlled directly by environmental light. In an earlier study, mice were exposed to either alternating illumination or constant illumination for 70 days and observations made on pinealocyte size and Golgi complex size.[60] Under continuous lighting, a significant reduction in the size of both was observed, while many of the mitochondria appeared swollen with a reduced number of cristae under these conditions.

The melatonin content of the pineal gland in 36 inbred strains of mice was measured at specific intervals during the day and night in mice kept under a 12-hour light/12-hour dark cycle.[61] The findings were somewhat curious. They demonstrated that in only five inbred strains (in two commonly used strains, C3H/He and CBA/Ms) and in three wild-derived strains (Mol-A, Mol-Nis, and MOM) was the pineal melatonin content higher during the night and lower during the day. In the other 31 strains examined, no detectable levels of melatonin were present at any of the times examined. This complemented another study in which different strains of mice were examined in an attempt to explain why no melatonin was detected in the pineals of a small number of strains while it was clearly present in all wild mice studied and in the majority of other strains of mice.[62] These researchers found that in several inbred strains of laboratory mice, such as C57BL/6J, no detectable amounts of melatonin were found in their pineal glands. They found that the melatonin deficiency noted in these mice was due to mutations in two independently segregating, autosomal recessive genes. In these strains, there was no evidence of enzyme activity involving either of these genes.

A further study was carried out to investigate the effect of melatonin on the reproductive cycle of male mice.[63] This study appeared to indicate that the pineal gland was of minimal significance in reproduction in the mouse, as blinding and olfactory bulbectomy did not result in testicular involution within 3 months of these procedures. In another study, it had also been noted that pools of serotonin were found in the pineal gland of the mouse.[64] It was suggested that the pinealocytes were the recepto-secretory elements of the mammalian pineal gland. This was one of the earliest studies strongly indicating that the pineal gland was in fact a diffuse endocrine organ. In a more recent study, it was stated that repeated subcutaneous injections of melatonin led to a decrease in the number and volumetric density of lysosomes.[65] This ultrastructural study indicated that melatonin influenced the secretory activity of the pinealocytes and demonstrated that they participated in a complex secretory regulating mechanism.

MOLECULAR STUDIES THAT HAVE BEEN UNDERTAKEN WITH MICE TO INVESTIGATE PINEAL DEVELOPMENT AND MORPHOLOGY

Very few studies appear to have been undertaken using transgenic mice to investigate the factors that influence pineal development and function. The findings from two representative articles are presented here.

One study attempted to investigate the molecular basis of the circadian rhythm using transgenic mice generated from C57BL and C3H mice.[66] These glands were isolated at 5 and 10 days after birth and from adult mice. The following were studied: S-antigen, serotonin, and dopamine-β-hydroxylase (DBH) immunoreactions. The latter was used to study sympathetic innervation of

the gland. The pineal gland was found to be more than two times larger in the C3H than in the C57BL adults. This difference was evident with regard to both the superficial and the deep parts of this gland. This difference, albeit to a lesser degree, was also evident in the postnatal mice. The S-antigen immunoreactivity was more intense in the adult C3H than in the C57BL mice, while the serotonin immunoreactivity was similar in the two strains at all of the stages studied, although more pronounced in the adult mice. The DBH immunoreactive area was also greater in the C3H mice. Both S-antigen and serotonin immunoreactive cells were also found in the habenular (epithalamic) region, but far more were present in the C3H mice. It was suggested that the difference in pineal morphology observed might have been related to the well-known difference in melatonin formation in these two strains. It appears that C3H mice produce large amounts of melatonin during the night, whereas C57BL mice, because they lack hydroxyindole-*O*-methyltransferase that converts N-acetyl-serotonin into melatonin, are consequently unable to produce melatonin.

A more recent study demonstrated that the homeobox gene *Otx2* (orthodenticle homolog 2 (Drosophila)) was essential for cell fate determination in retinal photoreceptors and for the normal cellular development of the pineal gland in mice.[67] Deficiency of *Otx2* converted the differentiating photoreceptor cells into amacrine-like neurons, while there was a complete lack of pinealocytes evident in the pineal glands. This study indicated that *Otx2* was of critical importance in the differentiation of retinal photoreceptor cells and for the normal development of the pineal gland. Earlier studies had demonstrated that homozygous null (–/–) mutant embryos lack the forebrain, midbrain, and anterior hindbrain[68] while initial anterior neural development requires *Otx2* in visceral endoderm. It has therefore been suggested that *Otx2* probably has a later role in the axial mesoderm and/or the neuroectoderm for the specification of the anterior-posterior (AP) identity.[69] *Otx2* heterozygotes display microphthalmia or anophthalmia, indicating that *Otx2* is involved in retinal development.[70]

FEATURES OF THE PRENATAL PINEAL GLAND OF THE SHEEP

The histological structure of the pineal gland in four groups of sheep embryos isolated at 27–69, 70–97, 98–116, and 117–150 days of gestation (approximately full term) was studied.[71] The first evidence of a pineal primordium was noted at about 30 days of gestation, and proliferation of the pineal parenchymal cells was seen shortly afterward. By about 98 days the pineal recess was obliterated, while the first evidence of the transition from pinealoblasts to pinealocytes was recognized at about 118 days of gestation. An increase in the overall volume of the gland was also seen at about this time, and it was noted that the parenchyma of the gland was principally made up of pinealoblasts, interstitial cells, and pigment-containing cells. It was also noted that the gland was by this stage becoming subdivided into lobes due to the invasion of its substance by fibrous septae. Capillaries were also present over the entire surface of the gland, and these were also particularly abundant in its medullary zone. Vasoactive intestinal peptide-positive fibers were also now distributed around the blood vessels within the gland, particularly around the pinealoblasts.

In an earlier study of similarly staged embryonic sheep material, pinealoblasts were first distinguished at about 54 days of gestation and were still recognized up to the time of birth.[72] It was suggested that these cells contained all of the organelles required for hormone synthesis. The population of interstitial cells was first recognized at about 78 days of gestation and also persisted until the time of birth. Relatively few of these cells were observed, and they tended to be located in the perivascular spaces of the gland. From about 118 days of gestation until birth, a third population of cells was distinguished that also differed morphologically from the pinealoblasts. These cells contained a large number of pigment granules of various sizes and shapes. The pineal glands at this stage of development displayed considerable evidence of neural innervation and vascularization. It was suggested that this was indicative of the fact that the gland probably had an active secretory function during the latter part of pregnancy.

Further light and electron microscopic studies were undertaken on the pigment-containing cells.[73] It was noted that these cells represented over 60% of the pineal cells in embryos isolated at 54–92 days of gestation and over 80% of the cells from glands isolated at 98–113 days of gestation. However, in the group of embryos isolated from 118 to 150 days of gestation, the incidence of these cells dropped to nearer 25%. The pigment present was identified as melanin. Several types of pigment granules were distinguished that varied in their size and location within the pineal glands and indeed were noted in all of the interstitial and pigmented cells studied, although the highest incidence was observed in the latter group of cells.

In a subsequent study, ultrastructural means were used on similarly staged sheep embryonic pineal glands to determine the histological features of the cells that differed morphologically from the pinealoblasts after about 98 days of gestation.[74] These cells showed uniform ultrastructural characteristics similar to those of astrocytes within the central nervous system (CNS). When immunohistochemical means were used to analyze these typically Type II cells, several subtypes were distinguished. While all of these cells stained positive to phosphotungstic acid hematoxylin, only about half expressed glial fibrillary acidic protein. The vascular affinity of this subgroup of cells led to the formation of a limiting pineal barrier, and it was suggested that they may have had a functional involvement in the exchange of substances between the blood and the pineal parenchyma.

In another study, the pineal glands of three different age groups of sheep were studied: infants (1–6 months old); a pubertal and early fertile group (9–24 months old); and adults (36–60 months old). All the glands were isolated either during the day or night during the summer months, when maximum natural daylight was present.[75] Plasma and pineal gland melatonin levels were assayed, and gland weights were determined. All values were higher in the pubertal sheep than in the infants or adults. The heavier glands also contained a larger number of pinealocytes and interstitial cells and had a more substantial innervation and greater blood supply. No difference was, however, observed in the number of pinealocytes or interstitial cells present in animals that were euthanized during the daytime or at night, although their gland weight, nuclear size and plasma melatonin concentrations were all significantly higher at night than during the day.

DEVELOPMENT OF THE PINEAL GLAND IN THE HUMAN

According to O'Rahilly[76] and Fawcett,[77] the human pineal gland is first seen at about Carnegie Stage (CS) 15 (33–36 days of gestation) as a prominent thickening in the posterior portion of the roof of the diencephalon. During CS 16 and 17 (37–43 days of gestation), cells then migrate into this ependymal thickening to form a mantle layer within which its cells form a follicular arrangement that gradually becomes transformed into the cell cords seen in the adult gland. By CS 18 (44–46 days of gestation), a distinct anterior lobe is formed in which follicles are first apparent. At the end of the embryonic period (CS 23, at about 8 postovulatory weeks), the posterior commissure, habenular commissure, and subcommissural organ are all present. By the sixth month, the differentiation of the two distinct cell types that largely constitute this gland—the *pinealocytes* and the *interstitial cells*—have occurred. A gradual increase in the size of the gland occurs until the adult dimensions are reached, at about the age of 7. The only change that is evident after this stage is an increase in the prominence of the connective tissue septa. The two cell types noted by Fawcett correspond to the Type I and II pinealocytes indicated above.

In one of the earliest detailed studies of the development of the pineal gland in the human embryo and fetus, Gladstone and Wakeley[3] noted that anterior to the pineal diverticulum were two small evaginations. The apices of these were directed backward, and they were clearly seen at about 3 months of gestation (about 60 mm Crown-Rump [CR] length). The more posterior of the two overlapped the base of the anterior lobe of the pineal body, and it was suggested that this represented the dorsal sac of reptiles. It was suggested that the anterior of the two evaginations probably corresponded to the paraphysis of reptiles.

HISTOLOGICAL FEATURES OF THE HUMAN PINEAL GLAND

Møller carried out two detailed ultrastructural studies on the human pineal gland in fetuses of various gestational ages between about 3 and 6.5 months of gestation. In the first of these studies,[78] the cellular morphology and vascularization of the gland was investigated, while in the complementary study[79] the pineal glands were examined to investigate their innervation and the presence and location of junctional complexes.

In the first of these two papers, Møller[78] noted that in fetuses aged between about 3 months and 6.5 months of gestation the pineal gland consisted of an anterior and a posterior lobe. Virtually only pinealocytes (Type I cells), however, were observed, and relatively few neuroblasts (Type II cells) were seen. The pineal organ was seen to be well vascularized and innervated with numerous unmyelinated nerve fibers. It was further suggested that the morphological appearance of the capillaries indicated that a blood–brain barrier was already established. This finding was, however, contrary to that of others.[80]

A subsequent study was undertaken on similar material to investigate the innervation of the pineal gland. It was noted that free nerve boutons[80] from the intrapineal nerve that entered the gland from the brain from the subarachnoid space and via the pineal stalk were present throughout the pineal parenchyma.[81,82] Such fibers were only rarely seen in pineals from the younger fetuses but were relatively abundant in those isolated from the older fetuses. Such nerves either penetrated the surface of the gland or entered the parenchyma with the septa from the surrounding pia mater. No evidence was, however, seen that these nerves made contact with the pinealocytes, although they were often in close contact with the pinealocytes and frequently invaginated their cell membrane. It was noted that their boutons contained principally clear and a few dense core vesicles, but no evidence of noradrenaline could be demonstrated in them.

Nerve terminals were also frequently closely apposed to the neuroblasts, but no synapses between them were observed. An analysis was also made of the cell junctions present in this organ as well as the junctions located between the ependymal cells in the pineal recess. Various types of junctional complexes were found between the pinealocytes, such as gap junctions and desmosomes. Tight junctions connected the capillary endothelial cells, and this was believed to be indicative of the presence of a blood–brain barrier. Tight junctions were also present between the ependymal cells in the pineal recess and formed an extracellular barrier between the pineal gland and the cerebrospinal fluid.

In addition to the features described in the Type I cells, or *pinealocytes*, in the human, a feature noted in addition to the commonly encountered organelles was the presence of synaptic ribbons. These consisted of dense rods or lamellae that were arranged perpendicular to the cell surface and were usually surrounded by small vesicles. Such structures were found throughout the peripheral cytoplasm of these cells and did not appear to have any particular relationship to nerves, glial cells, or other pinealocytes. They are also reported to increase in number by a factor of two or three in the dark phase of the diurnal cycle. Occasionally aggregations of up to 20 may be present and are then referred to as *synaptic-ribbon fields*. Their function has yet to be established. By contrast, the *interstitial cells* are mostly located in the pineal stalk and are believed to be comparable to the neuroglial cells observed elsewhere in the CNS.

In the human gland, and in that of some other mammalian species, curious concretions called *corpora arenacea* or *brain sand* is present in the extracellular spaces. Their mode of formation and significance is also unclear, although their number is somehow linked to the metabolic activity of the gland. When the secretory activity of the gland is increased, such as during a short photoperiod, more concretions are formed, and conversely, when pineal activity is reduced, fewer concretions are formed.

PINEAL STUDIES IN OTHER MAMMALIAN SPECIES

A relatively small number of studies on the pineal gland were also carried out in other mammalian species, including the rabbit, the domestic pig, the cat and dog, the hamster, and the mink.

The development of the pineal gland in the *rabbit* was studied between postnatal days 1 and 120. It was noted that the morphological features seen were similar in many regards to those previously noted in the postnatal rat by Garcia-Maurino and Boya.[83] Shortly after birth, the pineal was highly cellular, with two readily identifiable cell types present: pinealocytes of Types I and II. By about the fifth postnatal day, it was also possible to distinguish a cortical from a medullary region of the gland, a feature not described previously in the rat. The cortex differed morphologically from the medulla in that neither rosettes nor cellular cords were present in this region of the gland. Rosette-like structures were not seen in the medulla beyond the 30th postnatal day, and cords consisting of Type II pinealocytes were not seen beyond the 90th postnatal day, by which time the gland was believed to be morphologically mature.

In an immunohistochemical analysis of the pineal gland in the *domestic pig*, the pineal glands were isolated from newborn, 21-day-old, and 7-month-old pigs. Antisera to somatostatin and prosomatostatin were used to study the presence of these substances within cells and in different regions of the gland.[84] Nerve fibers and cells throughout the gland were displayed using this methodology, while the highest density was observed in the proximo-ventral and central part of the gland. Two principal sources of somatostatin were recognized—within the nerve fibers and intrapineal neurons or specialized pinealocytes. A significant difference was observed between the different age groups studied, with the lowest density of somatostatin seen in the adults compared with that observed in the younger animals.

A number of studies involving the pineal gland of the *dog* have been undertaken, all by members of the same group. In the earliest of these, the postnatal development of this gland was studied by light microscopy from the first postnatal day until maturity.[85] During the first postnatal week, large numbers of immature parenchymal cells, many of which were in division, were seen. Pigmented cells were also present both in the pineal gland and in extrapineal nodules. By the 20th to the 30th postnatal day, all of the cell types seen in the adult gland were already present. It was also noted that in the adult, the length of the gland had increased by a factor of four compared to the size present in the neonatal period. In a complementary study, the ultrastructure of the pineal gland was investigated. This showed that pinealocytes and pigmented cells were first recognized on the fourth postnatal day and pineal astrocytes by the 10th day. Immature cells were still evident in the glands of 1-month-old dogs.[86] In a subsequent study involving both dogs and *cats*, the expression of two glial antigens was displayed using immunohistochemical methodology.[87] In both species, VIM immunopositive cells were already observed during the first few days after birth, while GFAP expression was first observed during the second postnatal week. In the adults of both of these species, a considerable number of stellate cells—later identified as astrocytes—that were immunopositive for both of these antigens were seen.

In one of the few articles in which *hamsters* were studied, the development and morphological relationship observed between the superficial and deep parts of hamster pineal glands was described.[88] In this species, the gland begins to separate into these two parts during the first few days after birth, and separation is usually complete by day 12, although at all stages the morphology of the two parts is similar. By 20 days of age (at about the time of weaning) elevations in melatonin are observed during the night, but these, as in other species, can be abolished by exposure of these animals to continuous light.

In earlier studies using blinded hamsters and others exposed to short daily photoperiods, the pineal gland was shown to be capable of secreting a gonadotrophin-inhibiting substance, although its exact nature was unclear.[13] In a subsequent study, it was demonstrated that the gonadal atrophy involving both the uterus and ovaries that occurs after hamsters are blinded may be reversed following pinealectomy.[12] This is also observed in blinded male hamsters where testicular atrophy may be reversed following pinealectomy. The inhibiting substance produced by the pineal does not appear to completely inhibit the action of exogenous gonadotrophins (e.g., follicle-stimulating hormone [FSH], LH) on the ovary, as both the development of follicles and ovulation occurred, nor did this completely interfere with the action of estrogen on the uterus. However, the response of the

ovaries was greater than that of the uteri, and it was unclear whether the pineal–derived inhibitory substance interfered with the action of estrogen on the uterus.

Another species whose pineal gland has been studied in considerable detail is the *mink*. Under natural conditions, both the male and female display marked cycles in body weight, gonadal activity, molting periods, and hormonal patterns synchronized by annual changes in day length. Similarly, the duration of pregnancy also depends on the length of the daily photoperiod. In one study,[89] the role of the pineal gland and melatonin in conveying photoperiodic information to the target organs was described. Following bilateral ablation of the superior cervical ganglia, the mink became unresponsive to artificial manipulations in the daily photoperiod. Pinealectomy in females desynchronized some of these features, while injections of melatonin reproduced the effect of short days on hormonal secretion during the period of delayed implantation. Injections of melatonin also had a similar desynchronizing effect in males. It was suggested that in mink these findings supported the hypothesis that all photoperiodic signals were conveyed by the pineal gland but that the pineal did not modify these events once they were initiated. Similar findings had previously been reported in ferrets.[90,91] In white-tailed deer (*Odocoileus virginianus borealis*), the pineal also appears to influence antler growth.[92,93]

POSSIBLE CLINICAL APPLICABILITY OF MELATONIN BASED ON SOME OF THE EXPERIMENTAL ANIMAL FINDINGS

In a relatively recent review, Anisimov discussed a possible relationship between pineal function and mammary carcinogenesis.[94] He drew attention to the fact that inhibition of pineal function following pinealectomy, or following exposure to a constant light regimen, was capable of stimulating mammary carcinogenesis in rodents, whereas light deprivation inhibited the onset of this condition. It had earlier been demonstrated that treatment of pinealectomized rats kept in various lighting conditions with melatonin reduced the incidence of carcinogenesis. Attention was drawn to the increased risk of mammary carcinogenesis that had been noted in night shift workers, flight attendants, and other female shift workers, and a decreased risk in blind women. Based on these findings in rodents, it was suggested that treatment with melatonin might be a useful means of preventing breast cancer in women at risk.

While melatonin is now widely used, for example, for the treatment of jet lag, slowing or reversing the effect of aging, and enhancing immune function, there is little information on its safety, although this agent is now available without a prescription in some countries. No clinical trials have yet been undertaken to assess its clinical safety.

BIOCHEMICAL STUDIES ON FETAL AND NEONATAL ANIMALS

In a study on the influence of pineal hormones on sexual differentiation in the rat, Mess and Rúzsás[95] reported the findings of others on the functional capacity of the pineal gland in the fetal period. They noted that a number of studies had been carried out to investigate the secretory activity of the pineal gland during the fetal period. Owman,[96] for example, had noted that there was considerable biochemical evidence of secretory activity during the fetal period in the rat and suggested that it might even be more active in this regard than in the adult. This possibility was confirmed by later studies in the hamster[97] and the Malaysian rat.[98] While similar findings were not observed in the human fetus, Møller believed that the fetal pinealocytes possessed all of the organelles necessary for hormone synthesis to occur, although he was unable to demonstrate the presence of secretory granules in his material.[78] He argued, however, that the rich vascularization and innervation of the pineal combined with the existence of tight junctions between the ependymal cells argued in favor of a secretory role of the gland in early intrauterine life.

Subsequent biochemical studies indicated that the onset of active pineal indoleamine secretion occurred at a later period and that no demonstrable serotonin content was present until at least

a week after birth in the rat. Of particular interest was the finding that adult levels of this substance were reached by three weeks after birth. Dihydroxyphenylalanine (DOPA) decarboxylase and monoamine oxidase were first found in rats after about 1 week of age and reached adult levels after the 30th postnatal day.[99] Others[95,100] had noted that the rhythmic changes in pineal enzyme activity of the gland and the time of onset of its postnatal activity could be influenced by exogenous neonatal hormonal treatment, for example, by thyroxine injected during the first 3 days after birth. This accelerated rhythmic changes in pineal enzyme activity, while an injection of hydrocortisone acetate retarded the developmental decline in daytime serotonin-N-acetyltransferase activity. When pinealectomy was performed in male and female rats at 1 and 5 days after birth, no influence was observed in the reproductive capacity of rats of either sex following this procedure. In the females, however, reproductive maturation occurred 8–9 days earlier than normal, as evidenced by the premature opening of the vaginal membrane.[101]

CELLULAR COMPONENTS THAT CORRELATE WITH THE HORMONAL FUNCTIONS OF THE PINEAL GLAND

It has long been known that the pineal gland contains cells whose morphological features share some of those that are observed in retinal photoreceptors. The cells are light sensitive and are believed to play a critical role in the control of diurnal rhythm. In the rat pineal, granular vesicles in the terminal boutons contain noradrenaline and probably serotonin.[102] The latter is known to stimulate endothelial cells to contract, and this may be a means of regulating blood flow in the pineal. It is particularly relevant to note that serotonin is the precursor of melatonin. Serotonin is also a neurotransmitter substance derived from the amino acid tryptophan. Within the pineal gland, serotonin is acetylated and then methylated to yield melatonin. The synthesis and secretion of melatonin is dramatically affected by exposure of the eyes to light. Thus, serum concentrations of melatonin are low during the daylight hours and increase until they reach a peak during the dark.

These receptor cells secrete melatonin and act as neuroendocrine regulators. While this is probably the most well-known function of the pineal gland, it has relatively recently been realized that this gland also plays a critical role in a very wide range of other activities. Two other antigonadotrophic substances have also been isolated: arginine vasotocin from the human fetal pineal[103]; and a small polypeptide in the bovine gland.[104] Other studies have revealed that the electron-dense cored vesicles store both monoamines and polypeptide hormones,[105] the release of which appears to require sympathetic innervation. It is now clear, for example, that the pineal gland is an endocrine organ that plays a principally regulatory and largely inhibitory role in controlling the activities of the other endocrine organs.

THE ENDOCRINE FUNCTIONS OF THE PINEAL GLAND

While formerly believed to be a phylogenetic remnant, the derivative of the "third eye" of the lower vertebrates, the pineal is now considered to be an important endocrine gland that regulates probably all of the other endocrine organs, including the gonads, although its principal effect appears to be inhibitory. Some of the pineal amines influence circadian rhythms in many mammalian species. This is seen in the rat, where an endogenous circadian oscillator located in the suprachiasmatic nucleus of the hypothalamus appears to govern the cyclical behavior of this gland.[8]

Numerous studies[106,107] have now confirmed that the principal hormone released by the pineal gland is melatonin, although, as indicated previously, this gland also secretes a number of other hormones. The biological effects of melatonin are considerable (http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/otherendo/pineal.html). These particularly include effects on reproductive function in some species, where its principal effect is antigonadotrophic. It inhibits the secretion of LH and FSH from the anterior pituitary, mostly via its effect on the hypothalamus. This substance has

some effect on normal sleep patterns, although its exact role is unclear. It also influences the breeding season in many mammalian species and has been used specifically for this purpose, for example, in inducing certain breeds of sheep that normally only breed once per year to have two breeding seasons, by treatment with melatonin (http://arbl.cvmbs.colostate.edu/hbooks/pathphys/endocrine/otherendo/pineal.html).

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8 Development of the Mammalian Gonads and Reproductive Ducts during the So-Called "Indifferent" Stage as Well as during the Fetal and Neonatal Period

INTRODUCTION

This chapter concerns the development of the gonads, the ovaries and testes, and the reproductive duct systems in the two sexes during the period when gonadal differentiation is being achieved. By this means, it is possible to draw attention to the so-called "indifferent" stage of organogenesis of the gonads. This is when the exclusively morphological and histological features of the gonads do not allow them to be definitively recognized as either an ovary or a testis.[1,2] This equally applies to the genital duct system that is also at the indifferent stage until exposed to hormonal stimulation secreted by the gonads. These direct the components of the reproductive tract, and later the external genitalia, toward maleness if in the presence of at least a single Y chromosome or if in its absence in the "neutral" direction toward femaleness. Clearly, an analysis of the genetic sex of the individual would allow the fate of the indifferent gonad to be established, but for most purposes this information is neither needed nor, in most cases, likely to be available to the observer.

In all of the mammalian species so far studied, two sexes are present: the male and the female. This is determined at conception by the genetic sex of the individual, whether they possess an XX genetic constitution and will therefore give rise to a female or an XY sex chromosome constitution and, under normal circumstances, would be destined to develop as a male.[3] The situation in non-mammalian species varies considerably and is mentioned only in passing, as this is generally outside the scope of the present text, whose principal remit is to consider the factors involved in *mammalian* embryogenesis and organogenesis. It is, however, of interest to note that in certain submammalian species, such as in certain reptiles, the temperature at which the egg is incubated is what determines the sex of the resultant embryo.[4] In reptiles, once established, the sex of the individual is usually fixed. In certain species of fish, and snails, however, the sex, once determined, may change during their lifetime. In certain fish, the sex may be changed following the oral administration of sex steroids.[5] For example, in the medaka fish (*Oryzias latipes*), when a white female (XrXr) is mated with a heterozygous orange-red male (XrYR), the progeny are white daughters and orange-red sons in more or less equal numbers, and these can be used as markers of the sex genotypes XX and XY. Complete and functional reversal in sex differentiation in the XrYR genotype has been accomplished through the action of estrogens (estrone or stilbestrol) administered orally.[6,7] Sex reversal may be induced when the gonad is at the indifferent stage and continuing until the stage of differentiation. Thus, androgen-induced sex reversals of XX genotype fish have typical male traits and functions,

despite the absence of the Y chromosome.[8] However, for reasons indicated earlier, this undoubtedly extremely interesting and complex issue is beyond the scope of the present chapter.

A very considerable literature has been written over the last few years on the molecular factors believed to play a critical role in sex determination in mammals. However, as this field is developing at such a rapid pace, it is also for obvious reasons impossible to describe the most recent developments in this field. It is for this reason that only the earlier and consequently the most well-established observations are described here. As in previous chapters, most readers are likely to be interested in the analysis of the normal sequence of events observed in the *mouse*. This is because this species provides the majority of the animal models of human diseases. It is therefore of critical importance that an adequate overview is provided here of the histological features that may be observed during embryogenesis and organogenesis in the human and in the mouse. Clearly, an analysis of the sequential changes observed in the human embryo and fetus is required to provide the necessary background information before observations are given on the comparable events observed in other mammalian species, but particularly in rodents. The latter are based on as detailed an analysis of the literature as is thought reasonable to adequately cover this topic.[9-13]

With regard to the factors that influence the normal development of the gonads, the available background information tends to be somewhat more diverse than is the case with regard to the specific endocrine organs previously described. It is therefore for this reason that a more selective account of the earlier references is provided here in the hope that this will give an adequate overview of this topic appropriate for the interpretation of the early development of both the ovary and the testis.

In the case of mammals in general, it has long been established that the definitive sex of an individual is determined at conception and is dependent on whether the egg is fertilized by an X-bearing or a Y-bearing sperm. The sperm is therefore described as being *sex-determining*. If the former, then a female individual is the usual outcome, and if the latter, a male individual is usually induced. Recent information on this topic suggests that the only gene so far determined that plays a critical role in this process is the *Sry* gene, which is said to be present in most mammals.[14] To achieve an equal dose of the X-linked genes in the two sexes, the system of *X inactivation* has been established. Details of the basic underlying mechanisms involved have also long been described (see also in appropriate section below).[15-17] While it appears that not all genes on the X chromosome are subject to X inactivation, it has been suggested that X-linked gene dosage may contribute to differences between the sexes.

In the mouse (Figures 8.1–8.4 illustrate the key features of these organs), the phenotypic sex cannot be established until about E12–12.5 from the analysis of histological sections of either the gonad or of the components of the reproductive tract. This is the first stage when the *male* gonads possess certain characteristic features. In all of the mammals so far described, including the human, gonadal differentiation is first clearly recognized in the male, with the first evidence within the testes of "striping" due to seminiferous tubular and duct differentiation. By contrast, their absence in the female at this time provides one of the earliest means of identifying the ovarian phenotype. It should be noted that unequivocal evidence of the ovarian phenotype is not recognized in the mouse until several days after sex determination has been established morphologically in the male, at about E13–13.5. In the human embryo, testicular differentiation is first clearly recognized at about 47–48 to 50–51 dpc,[1,18] while the ovary is first unequivocally recognized some considerable time later, at about 16 weeks of gestation. Differentiation of the associated internal genital duct system, and later the external genitalia, occurs at an even later time, principally as a result of the influence of diffusible steroid and steroid-like products of gonadal origin.[19] The androgenic steroids originate in the interstitial (or Leydig) cells, while the hormone-like substances such as anti-Müllerian hormone (AMH; also termed Müllerian-inhibiting substance, or MIS) are secreted by the Sertoli or sustentacular cells located within the seminiferous tubules. For further details, see appropriate sections of the text. Oogonial meiosis is first seen at about 8 weeks of gestation in the human ovary, and this provides a means of allowing the ovaries to be recognized histologically, as no comparable process is observed in the testes until the time of puberty.

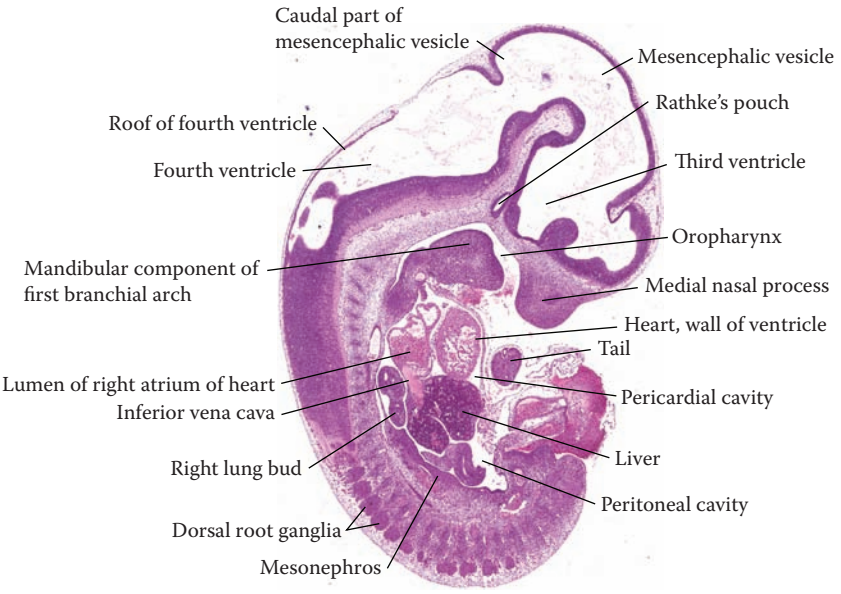

FIGURE 8.1 (E11.5_Fig8.1.svs) This represents a low magnification, approximately median sagittal section through the cephalic region of a mouse embryo (E11.5–12) that is stained with H&E.

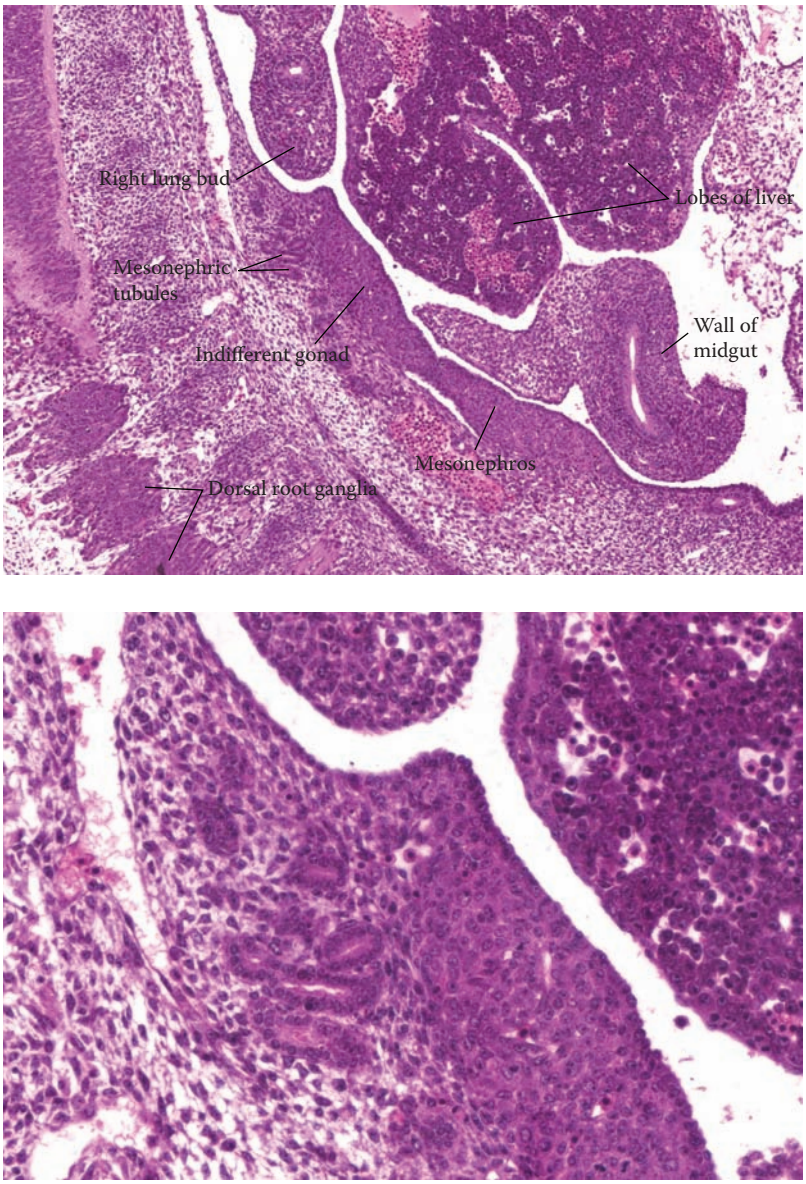

FIGURE 8.2 This is a medium (top) and high (bottom) magnification of Figure 8.1. The section is through the caudal region of the right side of the abdomen that displays the caudal part of the mesonephros and more rostral part of the indifferent gonad on that side. It is just possible to see the mesonephric tubules that are located just beneath the "indifferent" gonad within the urogenital ridge.

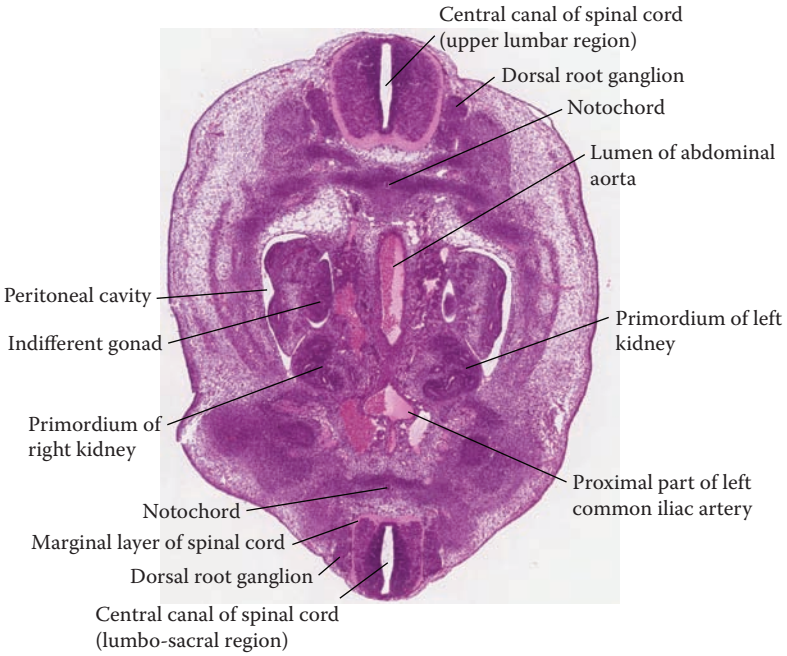

FIGURE 8.3 (E11.5–Fig8.3.svs) This represents a low magnification, transverse section through the caudal and posterior part of the future abdominal region of a mouse embryo (E11.5–12) that is stained with H&E. It is possible to note the presence of the primordia of the two kidneys still located in the pelvic region and have yet to migrate rostrally to their definitive position in the lumbar region of the embryo. The indifferent gonadal part of the urogenital ridge is particularly well seen on the right side of this embryo in this section.

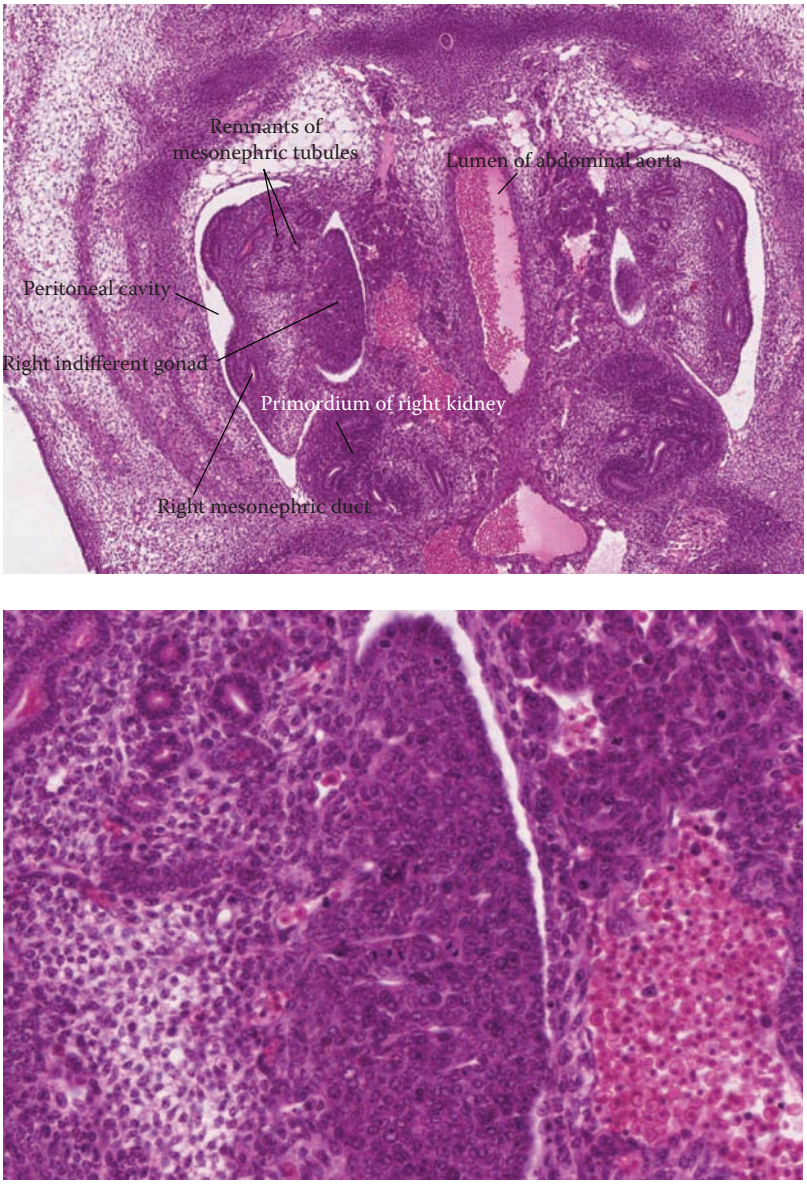

FIGURE 8.4 This is a medium (top) and high (bottom) magnification image of Figure 8.3. The medium magnification image illustrates the right indifferent gonad subjacent to which may be observed the right mesonephric duct and more rostrally the remnants of the mesonephric tubules. The lumen of the abdominal aorta is located just to the left of the midline in this section.

DEVELOPMENT OF THE UROGENITAL RIDGES AND GONADAL DIFFERENTIATION

Before the phenotypic sex is clearly established, the urogenital system is said to be at the so-called indifferent stage. In individuals of both XX and XY genotype, the urogenital system appears to be morphologically identical until *in the male* the indifferent gonad is induced, by the presence of the *Sry* gene, to develop into a testis rather than into an ovary. Before this occurs, however, the intermediate mesoderm of the nephrogenic cord differentiates to form the *pronephros* and subsequently the *mesonephros*. While the general consensus of opinion appears to be that the pronephros never functions in mammals, in the mouse this, the most rostral region of the nephrogenic cord, nevertheless develops a series of segmental tubules, each of which drains into a laterally located *pronephric duct*. The pronephros itself soon regresses in a cranio-caudal sequence. By about E10 in the mouse, its associated pronephric duct is taken over by the more caudally located mesonephros, thus giving rise to the mesonephric or Wolffian duct. The latter then acts both as the "inducer" and the "drainage" of the definitive mesonephros. In the human embryo, the pronephros develops during the third week and regresses during the early part of the fourth prenatal week.

Within the mesonephros, a segmental series of tubules, the mesonephric tubules, differentiates in a cranio-caudal segmental sequence, and these tubules then drain into the laterally located mesonephric duct. The latter then grows caudally and eventually drains into the cranio-lateral region of the urogenital sinus. Differences of opinion exist as to whether the mesonephros, both in the human and in the mouse, ever acts as a functional excretory unit. This clearly depends on whether the mesonephric tubules differentiate to form functioning glomeruli. In the human, as in the pig, sheep, and rabbit, the general consensus of opinion seems to be that the mesonephros does in fact function, while in the mouse this is not thought to be the case. While the mesonephros in the mouse usually develops tubules and may even occasionally develop Bowman's capsules, no functioning glomeruli usually form.[20] In some very rare examples in the mouse, where both the mesonephros and the *metanephros* (the definitive kidney) develop, morphological evidence of glomerular differentiation in both units is observed (Kaufman, unpublished observations), and in this extreme case mesonephric as well as metanephric glomerular function would appear to occur. However, it must be emphasized that, in the mouse at least, this situation tends to be the exception rather than the rule and has only very rarely been observed.

Under normal circumstances, the mesonephros, like the pronephros before it, regresses in a cranio-caudal sequence. Once the mesonephric duct grows caudally and later makes contact with the urogenital sinus, it sends off a cranially directed diverticulum, the *ureteric bud*, which is believed to induce the metanephric blastema, located at the caudal end of the nephrogenic cord, to differentiate into the definitive kidney. While the "excretory" component of the metanephros that is involved in the production of urine develops from its glomerular system, the majority of the derivatives of the ureteric bud give rise to the "drainage" system of the definitive kidney. Cases where the two systems both differentiate but fail to unite, either *isolated renal cysts* (which contain urine) form or, in more extreme cases, where multiple cysts are present, this may give rise to the condition termed *congenital polycystic kidneys*. When, as is usually the case, this condition occurs bilaterally, it is not compatible with viability. In these cases, no urine drains into the urogenital sinus, the future bladder. The condition is also invariably associated with *oligohydramnios*, where the fetus is surrounded only by a very small quantity of amniotic fluid, as the majority of the latter is formed from the urine.

Oligohydramnios may be due to a number of other causes. It may occur, for example, in association with esophageal atresia, and the latter is almost always associated with a range of characteristic limb and occasionally craniofacial congenital abnormalities. In other conditions, one or occasionally both kidneys may fail to develop, termed unilateral or bilateral *renal agenesis*, and the latter condition, i.e. bilateral renal agenesis, is often seen in cases of *sirenomelia* and *schizosomia*. Oligohydramnios may also be induced experimentally both in the rat[21,22] and

in the mouse,[23–26] for example, following deliberate amniotic sac puncture during the early post-implantation period. This experimental procedure is commonly associated with the induction of craniofacial and limb abnormalities in both of these species and may account for the occasional association between these abnormalities and chorionic villus sampling when this procedure is undertaken at a comparable period during human pregnancy.[27]

A substantial connection does in fact form between the mesonephros and the early gonad, and this develops into the rete testis in the male and rete ovarii in the female. Indeed, a number of experiments have demonstrated that the mesonephros has a strong influence on the developing gonad and on germ cell differentiation. When, for example, this connection is severed and the rete ovarii tissue is separated from developing mouse ovaries, it has been noted that if these ovaries are transferred subcutaneously into adult mice, further ovarian development is prevented.[28] A similar degree of influence of mesonephric tissue has also been observed in vitro in relation to the development of the early mouse ovary.[29] It is also commonly believed that both the Sertoli and Leydig cells may differentiate from mesonephric precursors (see following section).

PRIMORIDAL GERM CELLS

Gonadal differentiation occurs on the ventro-medial aspect of the mesonephros. The "genital" or future gonadal region of the urogenital ridge soon becomes invaded by diploid *primordial germ cells* (PGCs) of *yolk sac* origin. In the female, as noted previously, they possess an XX sex chromosome constitution, while in the male they possess an XY sex chromosome constitution. Relatively rarely, the primordial germ cells may possess a trisomic chromosome constitution, such as an XXY or XXX. The abnormal chromosome constitution of these germ cells clearly influences the subsequent development of the internal genital duct system as well as the external genitalia. The chromosome constitution of these germ cells also has other effects on the phenotype of these individuals. While the specific location of the PGCs within the wall of the yolk sac has yet to be definitively established, it is usually stated that they are first located in association with the extraembryonic *endodermal* cells of the wall of the yolk sac. Opinions, however, differ on this topic (see below). This contrasts with the earliest appearance of hematopoietic cells that are first clearly demonstrated in association with the extraembryonic *mesodermal* component of the yolk sac.[30]

The exact origin of the mammalian PGC has yet to be unequivocally established. What is clear, however, is that these cells are first clearly delineated within the wall of the yolk sac. The PGC are also characteristically round or ovoid cells that possess a larger diameter than the other cells in their immediate vicinity. These cells initially migrate from the wall of the yolk sac toward the base of the allantois. Such cells then migrate by active ameboid movement[31,32] with the aid of pseudopodia until they enter the site of the future gonadal region. It is unclear whether chemotaxis is involved in their lengthy migration from the wall of the yolk sac to the genital ridge, but this is thought to be a likely possibility.[31]

It has also been noted that fibronectin is present along the migratory pathway,[33] and it has been suggested that this may play a critical role in the migration of these cells from the yolk sac to the genital ridges.[34] In a number of rodent species, PGCs possess a unique surface glycoprotein that may also facilitate their migration, as it has been noted that different species possess slightly different glycoproteins. These appear to be lost on their arrival at the genital ridges.[35] What is particularly curious, in the light of the latter, is that when mouse germ cells isolated from embryos at the hindgut stage of development are transferred into the celomic cavity of a chick embryo, they appear to be capable of migrating to the chick's gonad.[36] When mouse gonadal ridges are retained in tissue culture, they appear to exert a long-range attracting effect on PGCs.[37]

The fact that PGCs attain their definitive location by ameboid movement was confirmed by the observations of Zamboni and Merchant, principally on the basis of the morphological characteristics of the PGC that were displayed during their migration, and that these cells also appeared to contain undifferentiated elements.[38] They noted that these cells appeared to lose their ameboid

features and assumed a much simpler structural organization shortly after they reached the genital ridges. It appears that the period of PGC migration is followed by a period of relative quiescence when mitosis is the only activity observed. Many of these divisions displayed incomplete cytokinesis. This was a feature that they had observed in both oogonia and spermatogonia in early sexually differentiated gonads.

This invasion of PGCs occurs at an early stage in gonadal differentiation, while the latter are still at the so-called "indifferent" stage. In the mouse, the first evidence of PGC migration into the future gonad is seen at about E10–10.5. This is most easily demonstrated when serial histological sections of the hindgut and urogenital ridge regions of appropriate stages of embryonic development in this species are stained to demonstrate the presence of intracellular *alkaline phosphatase* enzyme activity.[39] It should also be noted that other regions of the embryo at these stages, but particularly at the early limb-bud stage of development, also stain positively for the presence of intracellular alkaline phosphatase enzyme activity. These specific regions, including PGCs, also include the cells located just subjacent to the *apical ectodermal ridges* and the ventral (future "motor") regions of the neural tube.[40]

During the period when PGCs migrate from the wall of the yolk sac into the future gonads, up to about E13 in the mouse, these cells may also be found within the adrenal cortical anlage. After about E13, these cells are no longer found in this apparently aberrant location,[14,41-43] and it must be assumed that this is related to the fact that the adrenal cortical anlage is along the normal path of migration of these cells. By this means, all stages in the invasion of the gonadal or genital part of the urogenital ridge by mouse PGCs may be distinguished. Initially, all of these cells are located in the wall of the yolk sac, while in developmentally more advanced embryos all of the PGCs have normally reached the future gonadal region. At intermediate stages, some of these cells may be observed in the wall of the hindgut and, slightly later, within the dorsal mesentery of the latter. PGCs migrate by aggressive amoeboid movement into the gonadal ridges. PGCs in the mouse were first clearly identified and described by Chiquoine,[44] and over the years many others have subsequently confirmed these observations.[32,45-51] These cells have also been demonstrated by alkaline phosphatase enzyme staining in diploid parthenogenetic and in diandric and digynic triploid and in tetraploid fertilized mouse embryos.[52-54] They have also been demonstrated at comparable stages of development in the rat embryo.[55,56]

The ability to stain early somite and slightly more advanced stages of mouse development for the presence of intracellular alkaline phosphatase enzyme activity has allowed for estimations of the total number of PGCs present at sequential stages of their migration. By this means, it has been supposed that when these cells are exclusively located in the wall of the yolk sac, there may be up to about 100 PGCs present.[44-46] However, during their migration, their number increases dramatically, so that by about E13 when most of the PGCs have reached the genital ridges there may be as many as 10,000 PGC in this location.[47] As soon as PGCs reach the celomic epithelium of the genital ridges, they do not appear to wander elsewhere other than into the subjacent mesenchyme tissue.

In the human embryo, PGCs are first clearly recognized at the end of the third week of gestation by alkaline phosphatase staining.[57] In the human, PGCs reach the genital ridge by about the fifth postnatal week, when the premeiotic germ cells are then referred to as oogonia.[58] During weeks 5–7, during the indifferent stage, little is recognizable of the future gonad beyond a slight bulge on the antero-medial aspect of the mesonephros. By about 6–7 weeks, it has been estimated that the total number of oogonia present in the germinal ridge is about 10,000. By about 8 weeks, the total number of oogonia has increased to about 600,000, while by 3–6 months the total number present may be as high as about 7 million. From this point, the number diminishes, principally as a result of oogonial atresia.[57] After birth, there is a progressive decline in the number of ovarian follicles containing oocytes until menopause, when the ovaries are completely depleted of their oocytes.

The mechanism of depletion of oocytes during the third trimester of human pregnancy and during the postnatal period is principally through follicular rather than oogonial atresia, so that only about 1–2 million germ cells are present at the time of birth.[59] The total number of oocytes

decreases to about 300,000 by the onset of puberty, and less than 0.1% of these are destined to be ovulated during the reproductive life of the woman. Once formed, the primary oocyte persists in prophase of the first meiotic division until the time of ovulation. Meiosis is then resumed, and the first polar body is extruded. For a detailed description of the events that occur in association with folliculogenesis and ovulation, see Chapter 9 in this volume.

Occasionally, PGCs may migrate into ectopic sites rather than into the future gonadal region. While these germ cells usually perish outside the genital ridge, they occasionally continue to differentiate in these locations to form either benign teratomas or teratocarcinomas.[60,61] While such germ cell-derived tumors are relatively commonly seen in both the ovaries and testes, they are far less commonly seen in these ectopic sites. According to Alison and Morgan,[62] some of these tumors are found only during microscopic examination of grossly normal ovaries. Such tumors often contain one or more cysts, and these may be lined by cuboidal, enteric, or respiratory epithelium or by cornified stratified epithelium. Numerous differentiated and relatively easily recognized cell types are commonly present within these benign tumors. In the case of malignant teratomas, or teratocarcinomas, these are commonly characterized by the presence of poorly differentiated tissue. The major constituents of these tumors are immature nervous tissue and areas of hemorrhage and necrosis. Such tumors, when present in the ovary, may expand rapidly and extend through the ovarian bursa. In LT/Sv and in a few other strains of mice and in the human, many ovarian teratomas are believed to originate from the parthenogenetic development of oocytes.[63-67]

X CHROMOSOME INACTIVATION IN THE PRIMORDIAL GERM CELLS AND THEIR DERIVATIVES

While it has previously been noted that the genetic sex of the individual and subsequently the PGC is determined at the time of fertilization, it is often possible to recognize the presence of the sex chromatin (or Barr) body in the PGC once X chromosome inactivation has occurred. This mechanism by which only a single X chromosome is effectively active in a cell was first hypothesized by Mary Lyon, and it has been demonstrated that inactivation occurs approximately at the blastocyst stage, at about E3.5 in the mouse, or within a few cell divisions later.[68] This event occurs during mouse embryogenesis[69] and is particularly clearly seen in their oogonia.[70] While in most mouse tissues random inactivation of the X chromosome is observed, an apparent exception is observed in the extraembryonic membranes, where selective paternal X inactivation occurs.[71-74] In other tissues, selective inactivation of either the maternal or paternal X chromosome occurs during embryonic development.[75] Curiously, the same arrangement also appears to occur in the PGC of females where one X chromosome may be inactivated during their migration but may be reactivated so that *both* X chromosomes may be active by the time PGCs reach the gonads.[76] In oogonia in the human and in the mouse, only a single X chromosome is active,[77,78] but this may be reactivated during the leptotene stage of the first meiotic prophase so that the mature oocytes, in contrast to somatic cells, possess two active X chromosomes.[79,80] Andina[79] demonstrated that glucose-6-phosphate dehydrogenase (G6PD) activity was similar in oocytes from XX and XO fetal mice at early meiotic stages, but an XX:X0 ratio of 2:1 was approached at later times, thus suggesting reactivation of the G6PD locus. It appears that in the female PGC, the inactive X chromosome escapes methylation,[81,82] and this may provide the underlying explanation why they are capable of being reactivated during meiotic prophase. Once reactivated, the X chromosome remains active during oocyte growth and maturation.

In female individuals with an XO, or occasionally mosaic individuals with an XO/XX (i.e., sex chromosome constitution), the number of germ cells present in their ovaries may be severely depleted compared with the situation observed in females with a normal sex chromosome constitution. It is usually stated that in such individuals the total number of germ cells present at birth may be dramatically reduced compared with the situation in individuals with a normal sex chromosome constitution. The ovaries of XO individuals may be completely depleted of germ cells by the time they reach puberty. Similarly,

XXY males (usually termed Klinefelter syndrome males) are usually sterile because, by the time they reach puberty, their gonads are depleted of either all or usually the majority of their germ cells.

FATE OF THE PRIMORDIAL GERM CELLS IN THE MALE AND FEMALE

It is of considerable importance to appreciate that in the male PGCs migrate into the *medullary* region of the primitive gonad, in association with the *Sertoli* (or sustentacular) *cells*, and induce the formation of the seminiferous tubules. The cells not incorporated into the latter become located between the seminiferous tubules. Called *interstitial cells of Leydig,* these cells later secrete *testosterone*. The intratubular Sertoli cells play several roles in relation to the maturation of the developing spermatozoa and are also the source of the AMH. While PGCs are initially located close to the basement membrane at the periphery of the lumen of the seminiferous tubules, as spermatozoan cytodifferentiation progresses, the more differentiated cell types migrate toward the central or luminal region of the tubules. The sequential stages observed in relation to PGCs in the male are the primary and then the secondary spermatocytes, then the spermatids, and finally the morphologically mature spermatozoa. As these cells migrate toward the lumen of these ducts, they initially undergo the sequential changes associated with *spermiogenesis* and then those of *spermatogenesis* with the eventual formation of the readily recognizable spermatozoa. This sequence of events (termed the *spermatogenic cycle*) normally takes about 6 weeks in the adult.

While these cells have all of the characteristics of fully mature spermatozoa, they are still nonfunctional, in that at this stage they are still incapable of fertilizing an egg. To achieve this status, they need to be *capacitated*. This process normally occurs within the female reproductive tract, when the mitochondria within the midpiece of the spermatozoa become "activated." This is associated with a much more vigorous movement of the tail region of the sperm than was formerly the case. At about the same time, the head region of the sperm undergoes the so-called *acrosome reaction*. The latter allows the slow release of the proteolytic enzymes located within the *acrosome cap* and thus allows the sperm to penetrate through the glycoprotein shell (the *zona pellucida*) that surrounds the unfertilized egg. Capacitation may also be induced in vitro to allow in vitro fertilization (IVF) to be carried out. Under these conditions, capacitation is usually facilitated by the presence of a small amount of a high molecular weight substance, such as bovine serum albumin, in the tissue culture medium.

By contrast, in the female, PGCs come to lie within the *cortical* region of the developing gonad. Unlike the situation in the male, the total number of germ cells in the female appears to be fixed, with a maximum number being present in the human during the mid to late fetal period. At this time, there may be as many as 3–5 million or occasionally even more PGCs in the two ovaries. A rapid diminution in the number of PGCs occurs thereafter, as the majority undergo the process of atresia. In the human, for example, it has been noted that even by the time of birth the total number of germ cells present diminishes to about 1 million. By puberty, the number present is believed to be in the region of about 300,000, while by menopause the ovaries are completely depleted of their germ cells. During the reproductive life of the average human female, the total number of eggs ovulated only rarely exceeds about 300–400. In an individual with ovarian dysgenesis, the ovaries may be depleted of all of their germ cells by the time of puberty, and "streak" gonads usually replace the ovaries.

By the time of birth, in the normal human female a single or several layers of follicular cells surround all of the PGC to form a population of primordial follicles. Only after puberty, when ovulation occurs on a regular basis, do the follicular cells that are not a component of the follicular antrum become converted into the progesterone hormone-secreting cells of the *corpus luteum* (of either menstruation or pregnancy). Other intrinsic cells of the ovary become the estrogen-secreting *thecal* cells. Due to the high levels of circulating maternal (largely estrogenic) hormones at the time of birth, it has long been recognized that these stimulate some of the primordial follicles present within the fetal/neonatal ovaries to mature and to form secondary (or antral or Graafian) follicles.

A proportion of the latter are even induced to undergo ovulation, although these ovulated ova are clearly not destined to be fertilized.

GONADAL DIFFERENTIATION

Before considering some of the factors believed to influence gonadal differentiation, it is appropriate to note that the coelomic epithelium covering the future gonads was previously termed the *germinal epithelium*. This was because it was formerly believed that this was the site where the PGC originated. It is now believed that this epithelial layer has another function and differs in the two sexes depending on whether the gonad develops as a testis or an ovary. In the male, the primary sex cords that in the male are destined to form the future testicular cords are recognized at an early stage of testicular differentiation. These soon become separated from the coelomic epithelium as they invade the subjacent mesenchyme tissue and will give rise in due course to the seminiferous tubules.

In the female, the primary sex cords soon regress and are subsequently replaced by a second wave of epithelial invasion, called *secondary sex cords.* In both sexes, PGCs become surrounded in the male by the primary sex cords and in the female by the secondary sex cords. In the testis, PGCs become closely associated, within the seminiferous tubules, with the Sertoli cells, while in the ovary the granulosa cells take on a similar "supporting" role. Because of their similar function, it has been suggested that the Sertoli and granulosa cells are probably both of mesonephric origin.[83,84] They also appear to share a number of common structural and functional features. Both are epithelial in type, are involved in steroid production, and possess receptors for gonadotropins and growth factors.

FEATURES ASSOCIATED WITH EARLY OVARIAN DIFFERENTIATION

At the gross level, early ovarian differentiation is marked by the presence of mesonephric tissue located toward the central part of the gonad. The effect of the presence of the rete ovarii in this location is to concentrate PGCs toward the periphery of the future cortical region. The end result of this early phase of ovarian growth is a medullary region consisting principally of mesonephric cells, and these are surrounded by a cortical region containing large numbers of germ cells. At about the same time, the cells of celomic epithelial origin invade the subjacent mesenchyme tissue and gradually surround PGCs. In the female mouse, the germ cells enter the first meiotic prophase very shortly after the gonadal sex is recognized, although the follicles develop at a later stage. It is only then that steroid production is initiated. In the mouse and hamster, meiosis occurs at an early stage in ovarian development, and the germ cells tend to be distributed throughout the cortex. In other species, such as in the human, where early meiosis also occurs, the germ cells tend to be located in a well-defined part of the cortical region. In the rabbit, by contrast, well-defined cords of germ cells tend to be seen only in the innermost part of the cortical region, close to the rete region. While in the human, the germ cells enter meiosis at a relatively early stage of gonadal differentiation, minimal steroid production is observed at this stage.

FEATURES OF EARLY TESTICULAR DIFFERENTIATION

Within the early testis, the germ cells soon become enclosed within the testicular cords. This is associated with the differentiation of two distinct cell lineages: (1) the Sertoli cells, which become located within the testicular cords; and (2) the Leydig cells, which develop in the interstitial spaces between the testicular cords. While the former cells secrete AMH, the latter principally secrete testosterone. The earliest evidence of testicular differentiation appears to be the close association of the germ cells with the Sertoli cells and some somatic cells located within the testicular cords. It has been suggested that even at this stage the Sertoli cells express *Sry.* The testicular cords in the early human, in embryos with a CR length of about 14 mm, and in mouse embryos at about E13 are

arranged longitudinally in the gonad and appear to be connected to the rete testis. At about this time, or shortly afterward, an intact basal lamina forms around the testicular cords. This separates them from the surrounding interstitial tissue as well as from the majority of the mesonephric tissue.

Even by E14 in the mouse only a few connections are still present between the testicular cords and the epididymal tubules. Only when the germ cells are completely surrounded and located within the testicular cords are they then termed *prespermatogonia*. These cells start dividing mitotically and also initiate their morphological differentiation, a process called *prespermatogenesis*. This rapid increase in the number of these cells resulting from these mitotic divisions has been described in mice,[85] and it has also been noted that these cells as well as germ cells in the human fetal ovary are often connected by interstitial bridges.[86] It has also been noted that the germ cells that are not enclosed within the testicular cords invariably degenerate.

ORIGIN OF THE SERTOLI AND LEYDIG CELLS

Sertoli Cells

The origin of the Sertoli cells has yet to be definitively established. Evidence from certain studies suggests that the Sertoli cells might have a dual origin, from both mesonephric and celomic epithelial cells.[87] Similar evidence has also been obtained from the analysis of human material.[84] Others, however, based on evidence derived from the analysis of ovarian and testicular parenchymal cells in cattle had previously suggested that these cells may form from a single pool of cells.[88] Yet another study, based on the analysis of gonadal material in the rat, suggested that these cells may be derived exclusively from celomic epithelial cells.[89]

Whatever their exact origin, the Sertoli cells are, however, of considerable interest because they undergo mitotic division throughout the fetal and neonatal period but cease dividing once spermatogenesis begins. In the mouse, the highest rate of mitosis occurs at about E16, during the fetal period.[90] During the early stage of testicular differentiation, the Sertoli cells vary considerably in shape, with large numbers of germ cells in close proximity to them. Their cytoplasmic extensions then make contact with adjacent Sertoli cells but do not as yet form the blood–testis barrier characteristically seen in the adult. At later stages of Sertoli cell development, the appearance of their cytoplasm changes so that it becomes characterized by the presence of large amounts of rough endoplasmic reticulum. A dramatic increase in the output of testosterone has also been observed in the fetal pig at this time.[91] No information is presently available regarding the steroidogenic output of these cells at this time, although it was suggested that steroid production by the prospective Leydig cells probably accounted for the presence of steroids in the medium when fetal testes were maintained in tissue culture during this period.

The early demonstration by Jost[19] that the Sertoli cells were responsible for inducing the regression of the Müllerian ducts, due to their secretion of AMH, possibly related, albeit indirectly, to *Sry* production by these cells, was of considerable importance in establishing their role during early testicular development. The AMH produced by these cells in some species[92] is also believed to play a role in stimulating testicular descent during the neonatal period.

Leydig Cells

The principal role of the Leydig cells during the fetal period is the production of androgens, and these are first secreted shortly after the first evidence of gonadal sex differentiation is observed. While the origin of these cells has also yet to be unequivocally established, it has been suggested that they are probably derived either from mesenchyme cells[90,93,94] or possibly from mesonephric cells.[83,95,96]

Leydig cells proliferate at a slow rate during the early differentiation of the mouse testis and at an even slower rate after birth.[90] However, at about the time of puberty their number increases and

continues to do so until the time of sexual maturity. In the rat it has been noted that the rate of differentiation and increase in volume of the Leydig cells is related to a rise in testosterone levels,[97–99] and this appears to correlate with an increase in their number.[100] Similarly, it has been noted that differentiation of the testicular cords always precedes the differentiation of the Leydig cells. This observation would seem to support the idea that testicular cord formation is in some way crucial for Leydig cell differentiation. Gonadotrophins also influence Leydig cell differentiation so that when there are low levels of circulating gonadotrophins, fewer Leydig cells are present than are normally observed. Hypophysectomy is also associated with a depletion, or even a complete absence, of the number of Leydig cells present in the testis of the fetal rhesus macaque (*Macaca mulatta*).[101] When the level of gonadotropin secretion is higher than normal, as occurs in some sexual disorders, an increase in the number of Leydig cells is observed.[102]

FACTORS THAT INFLUENCE GONADAL DIFFERENTIATION

In the absence of the Y chromosome, the indifferent gonad develops in the direction of femaleness to form an ovary.[103] Furthermore, it has also long been appreciated that the inherent (or neutral) differentiation pattern of the mammalian embryo is to develop as a female. In the presence of even a single Y chromosome, however many X chromosomes are present, the gonad develops in the direction of a testis, as occurs in Klinefelter's syndrome, as previously discussed. This relationship with the Y chromosome and testis development was first observed in humans with Klinefelter's syndrome, and a complementary effect was observed in other individuals with Turner's syndrome.[104,105] A similar effect was also found at about the same time in the mouse.[106] It was not until 1990, however, that the factor on the Y chromosome associated with testis development was discovered: *SRY* in humans and *Sry* in mice.[107,108]

Little information appears to be available on the signals that initiate gonadal differentiation. It is, however, clear that these structures are of complex origin. Subjacent to the "germinal" or celomic epithelium is the mesonephric mesenchyme tissue, and this is associated with the so-called "drainage system" derived, in the male at least, from the mesonephric tubules and ducts.[2] The fact that in certain mutant strains of mice gonadal development occurs in the presence of degenerating mesonephric ducts and tubules, as occurs in mice that lack *Pax2*,[109] would appear to indicate that these components of the gonadal part of the urogenital ridge are probably not essential for gonadal differentiation. This is despite the fact that they play a critical role in its subsequent functioning.

Other studies have investigated the origin of some of the cells characteristically present within the testis, such as the Sertoli cells, and have demonstrated that they are likely to be of celomic epithelial origin.[110] Tumors derived from morphologically very similar cells may, however, occasionally be found in the mammalian ovary.[111] These tumors have various names (i.e., Sertoli cell tumors, adenoma testiculare ovarii, arrhenoblastomas, androblastoma, Sertoli's cell-like tumor, granulosa cell tumor, folliculoma lipidique, sex cord-stromal tumor) and have been particularly observed in the rat, although *arrhenoblastomas* have also been reported in other mammalian species, including in human females. These have been particularly noted during the third decade of life or in younger females but are rarely encountered, accounting for less than 1% of all human ovarian tumors. In a proportion of cases, the presence of these tumors is associated with virilization,[112,113] although this was not reported in the rat.[114] Occasionally these tumors may metastasize, often as multiple miliary nodules disseminated throughout the peritoneum.

Yet other studies[110,115,116] have demonstrated that there are two distinct cell lineages involved in gonadal development, although their exact origin has yet to be unequivocally determined. These form the "supporting" cells, or those that give rise to the steroidogenic lineage. It has been proposed that the origin of these supporting cells, that give rise to the Sertoli cells in the testis and to the follicle cells in the ovary, is probably from a single progenitor cell population whose fate depends on the environment in which they are located. These steroidogenic cells will almost certainly give rise to the Leydig cells in the testis and the follicular or thecal cells in the ovary (see above). Various

studies have indicated that other cell types, believed to be of mesonephric origin, may also enter the early testis—but after the Sertoli cells have initiated their differentiation. These cells have been described by both Martineau et al.[115] and Tilmann and Capel[116] and appear to play a role in seminiferous tubule formation.

It is also relevant at this stage to draw attention to the fact that the time that PGCs enter meiosis differs considerably between the two sexes and that some of the factors involved may also be species dependent. In the female, in all species studied, it appears that it is essential that both X chromosomes must be active for this to occur.[15,70] In a number of species, it appears that PGCs also need to be in close proximity to mesonephric tissue.[83] While it is essential in both the mouse and hamster that ovarian PGC enter meiosis in the presence of mesonephric tissue, in other species such as the rat and rabbit this does not appear to be an essential requirement. In the rabbit, for example, from the analysis of fetal ovaries maintained in tissue culture, it has been suggested that the pituitary gland may play a role in regulating the onset of meiosis.[2,117,118] Under normal circumstances, in the male, PGCs enter meiosis only at the time of puberty. However, under exceptional circumstances, PGCs in the male may enter meiosis at the same time as in the female. This occurs if they are situated in proximity to mesonephric tissue, for example under experimental conditions in vitro, when they are located outside the testicular cords[119] or when they are located within the adrenal gland.[83]

STEROID HORMONES PRODUCED IN THE DEVELOPING OVARIES AND TESTES

Ovary Steroid Production by the Ovary

As with the timing of the onset of meiosis, the time when steroid production is initiated in the fetal ovaries appears to be species specific. In the human, for example, the first evidence of steroid production is first noted during the last part of gestation,[120] whereas in other species, it occurs much earlier, usually shortly after the onset of ovarian differentiation. This difference in the timing appears to be related to the time when PGCs are enclosed within specific compartments such as the germ cell cords or within follicles. In primates where specific germ cell cords are not found, meiosis begins early in fetal life, although estradiol production is not observed until follicular development is established, that is, late in fetal life.[121] In the rat[122] and in the mouse[123] no steroids are produced when meiosis is first seen, only when the germ cells are enclosed in cord-like structures. Steroid production then diminishes but rises again when follicles form.[124] Ovarian steroid synthesis also appears to be dependent on gonadotropins in some species (e.g., in the mouse)[123] but to be independent of gonadotropins in other species (e.g., in the rat).[122]

Testis Steroid Production by the Testis

Unlike the situation in many females, in the males of all mammalian species so far studied, the testis is able to synthesize testosterone from acetate at a very early stage of gonadal differentiation.[125] The various steroids produced by the testis are particularly responsible for the differential development of both the internal reproductive duct system and the external genitalia (see following section). Equally, the androgens produced by the testis are believed to have a masculinizing effect on the brain during the critical early stages of brain development in many mammalian species. In the rat, and presumably in other species, it is not exactly clear whether testicular androgens or other factors play a role in the masculinizing of the brain.

It is certainly well established that AMH plays a critical role in inducing the regression of the paramesonephric duct in the male (see following section). It has also been suggested that AMH and possibly other testicular-derived substances may play a critical role in controlling testicular descent in some species (see below). While the factors that control steroid production in the testis have yet to be fully established, it is believed from certain experimental studies that gonadotropins may have a role to play in stimulating their production.[126]

FORMATION AND DIFFERENTIATION OF THE GENITAL DUCTS

It has long been recognized that the genital tracts in the male and female, while initially in close proximity to the mesonephros, soon diverge morphologically according to the genetic sex of the individual. In the male, the growth and differentiation of the mesonephric (or Wolffian) duct to form the "drainage" system of the testis depends on the presence of testosterone secretion. Under appropriate hormonal stimulation, the mesonephric duct gives rise to the appendix epididymis, the epididymis, the ductus (or vas) deferens, and the seminal vesicle. As noted previously, the mesonephric duct develops in a cranio-caudal direction and eventually makes contact and fuses with the outer part of the future trigonal region of the urogenital sinus. Regression of the paramesonephric (or Müllerian) duct occurs in association with the complementary secretion of AMH secreted by the Sertoli cells.

By contrast, in the female, in the absence of these hormones at this relatively early stage of development, it is believed that the paramesonephric duct is not "induced" to regress and, in due course, gives rise to the oviduct, uterus, and upper part of the vagina. Similarly, in the absence of the stimulatory effect of testosterone, the mesonephric duct eventually degenerates. It was formerly believed that the mesonephric duct either simply served as an "inducer" of paramesonephric duct formation[127] or that the latter formed following the longitudinal splitting of the mesonephric duct. This latter view is no longer accepted, as subsequent studies have indicated that the mesonephric duct appears to release epithelial cells that contribute to the developing paramesonephric duct as it grows caudally[128] and that in the rat at least, cell contacts are also present between Müllerian and paramesonephric cells. The latter appear to be disc-shaped thickenings between the adjacent plasma membranes. They also possess regions with fused areas of plasmalemmata over relatively short distances.[129] According to Gruenwald,[128] in the human embryo, a common basal membrane was present between the tips of these two ducts. This author was, however, uncertain whether the mesonephric duct actually contributed cellular material to the tip region of the paramesonephric duct in a way subsequently described by Dohr and Tarmann.[129]

As has been noted elsewhere, in rodents the Müllerian tubes remain as separate entities except at their caudal extremity, whereas in most primates, including the human, these ducts fuse across the midline to form a single median uterine structure.[130] The oviduct, by contrast, differentiates into three regions: the ampulla, the infundibulum, and the isthmus. In rodents, differentiation of the oviduct occurs principally after birth, while in the human, possibly because of its extended gestation period, it largely occurs during the midgestational period. While it appears that neither gonadotropins nor the ovary are necessary for the growth and differentiation of the female genital tract, this contrasts with the situation in the male where both gonadotropins and the testis are required for the development of the male reproductive tract.[131,132] These authors confirmed the presence of basic fibroblast growth factor (bFGF), also termed fibroblast growth factor 2 (FGF2), in both the mesodermally derived fetal and adult reproductive tracts. To study the possible role of this factor, explantation studies were undertaken, when it was noted that growth suppression of the male reproductive tract occurred in association with inhibition of differentiation of the epididymis when endogenous bFGF was neutralized with anti-bFGF. After 7 days of treatment, growth of the epididymis was impaired by 75%, and by 14 days, the epididymis was completely absent. These findings complemented earlier observations on the role of steroids and AMH on the growth and differentiation of the male reproductive tract.

While it appears that the development of the paramesonephric duct is not an estrogen-dependent process,[133] estrogen receptors have nevertheless been observed in the Müllerian ducts of fetal rats during the last part of gestation.[134] In the female, the Wolffian duct invariably degenerates because of the absence of androgens, and degeneration is first apparent shortly after gonadal sex differentiation. In the human, the effect is generally complete by the beginning of the third trimester of pregnancy.[135] Similarly, growth of the Wolffian duct occurs only when the genital ridges of fetal rats and mice are co-cultured either with testosterone or testicular tissue.

In the male, the most cranial part of the Wolffian duct develops into the epididymis, the intermediate portion develops into the ductus deferens while the most caudal portion forms both the ejaculatory duct and seminal vesicles. A number of the most cranial of the mesonephric tubules are connected to the rete testis and become the efferent ductules of the epididymis. At the caudal extremity, the seminal vesicle, in the human, arises from a dilatation of the Wolffian duct at about the 60 mm CR length stage,[136] while the most caudal part of the Wolffian duct gives rise to the ejaculatory duct. This enters the prostatic region of the urethra, one on either side of the prostatic utricle (see later). It has been suggested that the differentiation of the efferent ductules and the mesonephric duct derivatives in the male are stimulated by androgens secreted by the Leydig cells.[3] By contrast, in the human female, the paramesonephric ducts, although initially two columns of tissue, later canalize and differentiate to form a single continuous system consisting of the uterus and upper part of the vagina, associated with two oviducts, representing the rostral parts of the two paramesonephric ducts.[130] In nonprimates, only the most caudal parts of the paramesonephric ducts fuse together to form a single midline structure, although the extent of fusion tends to vary considerably between species.

The AMH, as noted previously, is a diffusible transforming growth factor β-related growth factor produced by the Sertoli cells.[19] Two other diffusible factors are believed to play a critical role in the development of the reproductive tract: testosterone, produced by the Leydig cells and insulin-like (factor) 3 (*Insl3*).[137,138] Testosterone, an androgenic steroid hormone, is responsible for the specific differentiation of the male reproductive tract, the male external genitalia, and male-specific regions of the brain. In the female, the hormones produced by the ovary are estrogen and progesterone. The Leydig cells also produce *Insl3*, and together with the testosterone that they also secrete, these substances play a role in stimulating the descent of the testes, culminating in their descent into the scrotum. The latter normally occurs in the human at or shortly before birth, being directed into the scrotum by the *gubernaculum testis*. By this means, it is the factors produced by the testis that play a critical role in inducing sexual differentiation in the male during the latter part of embryogenesis and throughout organogenesis.

Most of the information on the genetic factors that influence the differentiation of the intermediate mesoderm and urogenital ridge has been obtained from the analysis of mouse mutants. To date, this has principally supplied information on the development of the indifferent stage and not on the subsequent development of the ovary and testis.[14] Information on gonad differentiation has also been obtained from the analysis of the wide range of mouse mutants that have recently become available, and for additional information the interested reader should refer to one of the recent reviews on this topic.

It appears that a spontaneous point mutation in the androgen receptor gene has been observed in mice, termed testicular feminization (Ar^{Tfm}). In these XY mice, as occurs in XY humans with a deficiency in the same gene, testis formation nevertheless occurs. This appears to be in association with an inability of the "target organs" to respond to the presence of the androgens produced by the testis. In both species, a very similar phenotype is observed so that there is neither a female reproductive duct system nor male external genitalia formed. Lyon and Hawkes[139] first described this condition in the mouse, and Gaspar et al. recently defined its specific features in greater detail.[140]

The definitive location of the gonads in both species appears to be controlled by the cranial suspensory ligament (CSL) in the female and the gubernaculum in the male. In the mouse, testis descent occurs in two phases: The first occurs between E15.5 and 17.5, when the testis descends to the inguinal region, and the second phase occurs between 2 and 3 weeks after birth, when the testis eventually descends into the scrotum. This approximately coincides with the completion of weaning in this species. In the absence of *Insl3* bilateral cryptorchid testes are present, while the testes remain located within the abdominal cavity.[141,142] Failure of complete descent of the testes is also seen in the Ar^{Tfm} mouse, where the gonads are usually located in an intermediate position between the normal location of the ovaries and testes. In cases where Ar^{Tfm} mice were mated to *Insl3*-deficient mice, in the compound homozygous double-mutant mice, the testes were completely undescended and intra-abdominal and were located close to where the ovaries were normally found.[142]

In the human if, after birth, the testes remain abdominal in location, there is a finite risk that the germ cells will undergo a neoplastic change and develop into teratocarcinomas. This clearly does not occur in other mammals in which the testes descend into the scrotum only at or shortly before mating. It has also been suggested that in most species, AMH may be involved in controlling testicular descent into the scrotum.[92]

THE PRESENCE OF EMBRYONIC VESTIGES IN BOTH SEXES

The embryonic vestiges associated with the ovaries are believed to be in all cases homologous with structures associated with the testes. While in males these all possess an appropriate function, this is not commonly the case in the female. Complexities have also arisen because the nomenclature given to these structures in the female does not always relate to the homologous structures present in the male. Accordingly, Mossman and Duke[143] suggested that this should be the case, and they drew attention to the terminology of the rete (rete ovarii), the efferent ductules of the ovary, and the female ductus deferens (or Gartner's canal), all of which possess male homologs. The ovarian end of the female ductus deferens, for example, receives the efferent ductules and has been termed the duct of the *epoophoron*; it is the homologue of the epididymis. The most rostral end of this longitudinal tubule may end in a cyst-like expansion, termed the *hydatid of Morgagni* in the female. This remnant of the Müllerian duct is synonymous with the appendix testis in the male. The major portions of the efferent ductules are derived from the tubules of the middle segments of the mesonephros—these are collectively termed the *paroophoron* in the female and the *paradidymis* in the male. The rete ovarii is homologous with the rete testis and is located at the cephalic end of the hilar region of the ovary; it is believed that many human ovarian cysts may be derived from this structure.

In the male, a number of vestigial structures are also recognized. One of these is the *appendix testis* (also termed the *appendix vesiculosi epoophori*). This represents a remnant of the upper end of the paramesonephric duct (see above). The other vestigial structure represents the caudal end of the paramesonephric duct. This is variously termed either the *uterus masculinus* or the *prostatic utricle* (also termed *utriculus prostaticus* or *vagina musculina*). According to Hamilton and Mossman,[144] this caudal part of the paramesonephric duct may disappear completely, when the prostatic utricle may arise solely from the urogenital sinus and represent the sino-vaginal bulbs. The prostatic utricle does not normally possess a true uterine component but occasionally may form a diminutive uterus that may even possess bilateral uterine tubes.[145]

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9 The Ovary

INTRODUCTION

While the gross morphology of the female mammalian reproductive tract varies considerably between species, most noticeably between primates, of which the human represents an example, and the nonhuman primates, of which rodents represent examples, even within the latter group there are minor differences. This particularly relates to the structure of the rostral ends of the paramesonephric (Müllerian) ducts, in the arrangement of the ovarian bursae[1] and in the caudal end of the same system where the degree of fusion, or nonfusion, varies somewhat. Ferm[2] also briefly describes these minor differences. However, the principal difference between the primate and the nonhuman primate uterus is that the body of the uterus in the primate is represented by a single midline structure. This is formed by the midline fusion of the intermediate portions of the two paramesonephric ducts. During the early stages in the development of the latter, a substantial septum is present, showing the site where the two ducts are in the process of fusion. Occasionally, if fusion is incomplete, this may result in the development of a so-called septate or subseptate uterus. The presence of the latter may also be associated with a distinct and characteristic antero-posteriorly directed ridge that is located on the peritoneal surface in the region of the uterine fundus. In the human, this condition may occasionally be associated with the premature onset of labor or a transverse lie or breech presentation.[3] In the subprimates, two uterine horns are present, and these remain as two distinct structures caudally as far as the cervical region. At this level, it has been noted that slight variations exist even between the various rodent species.[4]

With regard to the gross morphology of the mammalian ovary, minor differences among various primates regarding this structure have also been reported—for example, between the morphology of the human fetal ovary and that of the rhesus macaque (*Macaca mulatta*). In particular, it has been reported that some of the features of the primitive cortex and medulla of this species of nonhuman primate are more distinct than comparable regions in the human ovary. Accordingly, there is a clearer relationship between its stromal and epithelial elements, and these differences persist throughout their prenatal development.[5]

LOCATION OF THE MAMMALIAN OVARIES

Initial practical observations are provided here on the location and gross anatomy of the ovaries in both the human and mouse (as an example of one of the members of the rodent family). This is particularly relevant if the ovary and/or the oviduct, utero-tubal junction, or uterus needs to be isolated during a surgical or experimental procedure. In some animals, a membranous capsule and bursa surround the ovary. If present, the former would have to be opened to allow direct access to the surface of the ovary, to the fimbriated os of the oviduct, or in some cases to the entire oviduct and/or the region of the utero-tubal junction. It is also relevant to be aware of the nature and location of the blood supply, lymphatic vessels, and nerves to this region. Similarly, variations exist between the appearance of the ripening follicles and the corpora lutea in living animals.

In the human, the ovaries are located in the sacral region or within the true pelvis. The possibility has been suggested that their location in the human may be related to the posture or gross morphology of the uterus. In the mouse, by contrast, in which there are two lengthy uterine horns, the location of the ovaries is significantly more rostral and is usually opposite the caudal portion

of the kidneys. In other mammals, such as in squirrels, the mink, dog, and horse, the ovaries may be located as far rostral as opposite the third or fourth lumbar vertebrae. The ovaries are also commonly found at this level in many species of rodents.

In general, it has been noted that the most caudal position of the ovaries are observed in species with a single uterus, particularly when this is associated with a short pair of uterine horns (or oviducts). This appears to be the normal arrangement in the primates, with the exception of the lemurs, which usually possess short straight bicornuate uteri.[1] It has also been noted that the location of the ovaries is not greatly altered during pregnancy, and the position of the mammalian ovary is far less variable than is that of the testis.[6] Furthermore, in all mammals, the ovary is attached either via the broad ligament or directly to the posterior abdominal or pelvic wall by a thin fold of peritoneum.

Broad ligament is usually used to describe the peritoneal attachment of the ovary, uterus, and oviduct is, as noted above. This is subdivided into the more caudal portion attached to the uterus, termed the *mesometrium*, and the more cephalic portion attached to the oviduct, termed the *mesosalpinx*. The cordlike thickening that marks the boundary between the mesometrium and mesosalpinx is the round ligament of the uterus (*ligamentum teres uteri*). A similar fibromuscular fold extends from the utero-tubal junction along the medial side of the broad ligament to the caudal pole of the ovary. This is termed the *round ligament of the ovary* (or *ligamentum ovarii proprium*). The two round ligaments of the ovary and uterus correspond to the *gubernaculum testis*.

THE OVARIAN BURSA

In the mouse, although not in the human, the ovaries lie within a membranous pouch that completely isolates them from the peritoneal cavity, and the oviducts are also located within them. As the bursa is complete in this species, the internal opening of the oviduct (usually referred to as the fimbriated os but also occasionally the infundibulum) lies within it.[7] Because of the apparently irregular "kinking" of the oviduct in the mouse, it is difficult to identify specific regions of it. The ampullary region, for example, is easily identified only when viewed through a dissecting microscope when it is dilated, due to the presence within it of the cumulus oophorus within which are the recently ovulated oocytes and the ovulated follicular fluid. This is also the region where fertilization normally takes place.

In the absence of the ovarian bursa, as is the situation in the human, this allows the possibility of transuterine migration of ova from one ovary to the opposite oviduct. This has occasionally been reported in cases where the oviduct on one side is blocked and when a tubal ectopic pregnancy is observed in the contralateral oviduct. Similarly, a uterine pregnancy may be associated with the presence of a corpus luteum (CL) on the side associated with a blocked oviduct. This also allows the possibility of an abdominal pregnancy should the fertilized egg be flushed rostrally through the fimbriated os into the peritoneal cavity rather than caudally along the oviduct and into the uterus, as normally occurs.[8,9] The close character of the mouse ovarian bursa has recently been used for selective introduction of genetic alterations into the ovarian surface epithelium.[10–13] In the rat, by contrast to the situation observed in the mouse, Kellogg has indicated that a small orifice connects the cavity of the ovarian bursa with the general peritoneal cavity,[14] while Clewe has demonstrated the lack of a peritoneal connection in the golden hamster.[15] In some species such as the cat, hyena, guinea pig, mole, and pig, the ovarian capsule is widely open, whereas in whales it is totally absent. In most species, the periovarial sac varies widely in the extent of its development. In the dog, bear, sea lion, and racoon, only a small opening exists in the wall of the sac.

Mossman and Duke[1] have drawn attention to the fact that very little is known about the function of the ovarian bursa. While it is generally stated that this bursa assures the entrance of the ovulated ova into the oviduct, it is relevant to note that many species that ovulate only a single egg possess no bursa whereas numerous polyovular species have complete bursae.

BLOOD SUPPLY TO THE OVARIES

In all mammals so far studied, the ovary invariably has a double blood supply. The principal arterial supply and venous drainage is from the ovarian artery and vein, and these are direct branches from the dorsal aorta and inferior vena cava, respectively. The origin of the arterial supply is from the upper lumbar region directly from the abdominal aorta usually just below the origin of the renal vessels. The left ovarian vein commonly drains into the left renal vein close to the inferior vena cava, while the right ovarian vein drains directly into the inferior vena cava. When the ovaries occupy a lumbar position, then the ovarian arteries run laterally from the aorta at a less acute angle than when the ovaries are pelvic in location. These vessels enter the mesovarium and then travel to the hilar region of the ovaries. In the human, the principal vessels usually enter and the veins leave the ovary at the hilar region, and this tends to be located near to its cephalic edge.

Relatively large arterial branches enter the ovary at its hilar region and branch repeatedly as they pass through the medulla. Because of the tortuosity of their course, these are termed the *helicine arteries* (or *arteriae helicinae*). From these larger diameter vessels, smaller branches pass into the cortex where they pass between the follicles and eventually break up into an extensive capillary network. The venous drainage of the various parts of the ovary is equally tortuous. It eventually empties into the large ovarian vein that is located at its hilar region. Usually, longer and finer diameter branches of the uterine vessels extend along the mesosalpinx to supply the oviduct, and these and the uterine vessels also send fine branches internally to anastomose with the ovarian artery. In the human, the ovarian veins break up to form a coarse plexus near the ovary, being similar in arrangement to the pampiniform plexus of the testis. Both of these vessels are derived from the venous plexus of the mesonephros. In the human male, in which the testis descends into the scrotum, the pampiniform venous plexus acts as a heat exchanger.

LYMPHATIC DRAINAGE

The ovary has a rich lymphatic plexus whose drainage is through the medulla by way of numerous large lymphatic vessels. Its drainage parallels the blood vascular supply but drains to the middle lumbar lymph nodes.[16]

NERVE SUPPLY

Various studies have been undertaken to investigate the nerve supply of the ovaries, and these appear to vary in their function in different species. In the human, the critical supply would appear to be the plexuses that run along with the ovarian arteries. These contain afferents from the 10th thoracic nerve and parasympathetic and sympathetic efferent nerves.[17] These nerves are principally formed by branches of the renal and aortic plexuses and are thus derived from the coeliac plexus. In the mouse, as well as in a number of other species Hill[18] has noted that when the nerve plexuses associated with the uterine vessels were cut, no effect was observed on the ovarian cycle and function. However, a marked effect was observed when the nerves associated with the ovarian vessels were sectioned or when he performed a thoracic vagotomy. While these nerves penetrate into the cortex of the ovary and are located around the follicles, it appears unlikely that they penetrate into the follicles.

When the pelvic nerves were sectioned in the rat, this severed the afferents responsible for the induction of pseudopregnancy (pseudociesis) but had no effect on ovarian function. Similarly, abdominal sympathectomy had no effect on either ovarian or uterine function.[19] In general, it appears that the ovary receives all of its neural innervation by way of fibers that run along with the ovarian vessels. While the afferents are from the lower thoracic nerves, both sympathetic and parasympathetic fibers are also believed to be present.

GROSS AND MICROSCOPIC APPEARANCE OF THE MAMMALIAN OVARY

The ovaries in the adult human vary considerably in size. In the adult human, they are generally oval in shape, varying between 2 and 5 cm in length with a width of between 1.5 and 3 cm and a thickness of about 0.5 to 1.5 cm. During the reproductive period, they normally weigh between 10 and 20 g with an average weight of about 14 g. Their normal location, attachments, and vascular and neural connections have been discussed previously. The ovary is described as consisting of three distinct regions. The outermost part of the ovary is termed the *cortical region,* and this is surrounded by the so-called germinal epithelium (see below). The ovarian follicles develop in the cortical part of the gonad. Its central medullary region consists of stromal tissue, while the hilar region is where the ovary is attached to the mesovarium. It is within this region that its vascular supply and neural elements are principally located, and it is this region that has the potential to become active in steroidogenesis and to form androgen-secreting tumours. In this location, the cells closely resemble the testosterone-producing Leydig cells of the testis.

The histologic features of the mouse ovary during embryogenesis are illustrated in Figures 9.1 through 9.8.

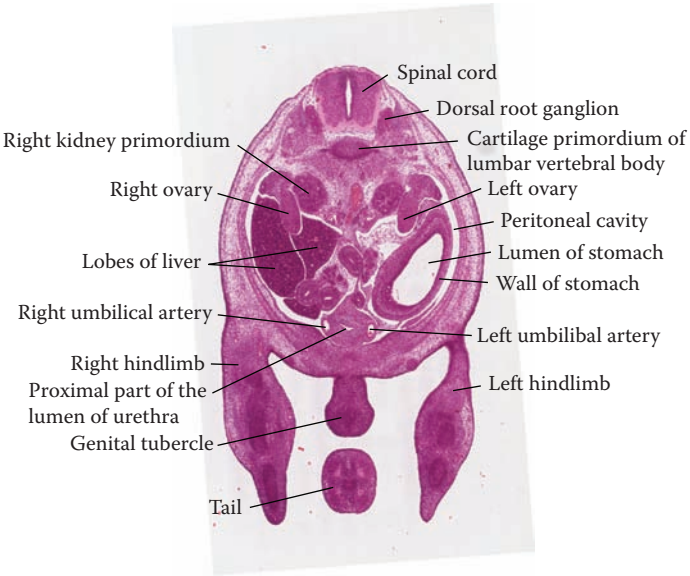

FIGURE 9.1 (E12.5_Fig9.1.svs) This represents a low magnification, transverse section through the midabdominal region of a mouse embryo (E12.5–13) that is stained with H&E, in which the right and left ovaries and the right and left kidney primordia are clearly seen. The lumen and wall of the stomach are also readily seen and appear to occupy much of the left side of the abdominal cavity, while the comparable space on the right side is largely occupied by the two lobes of the liver.

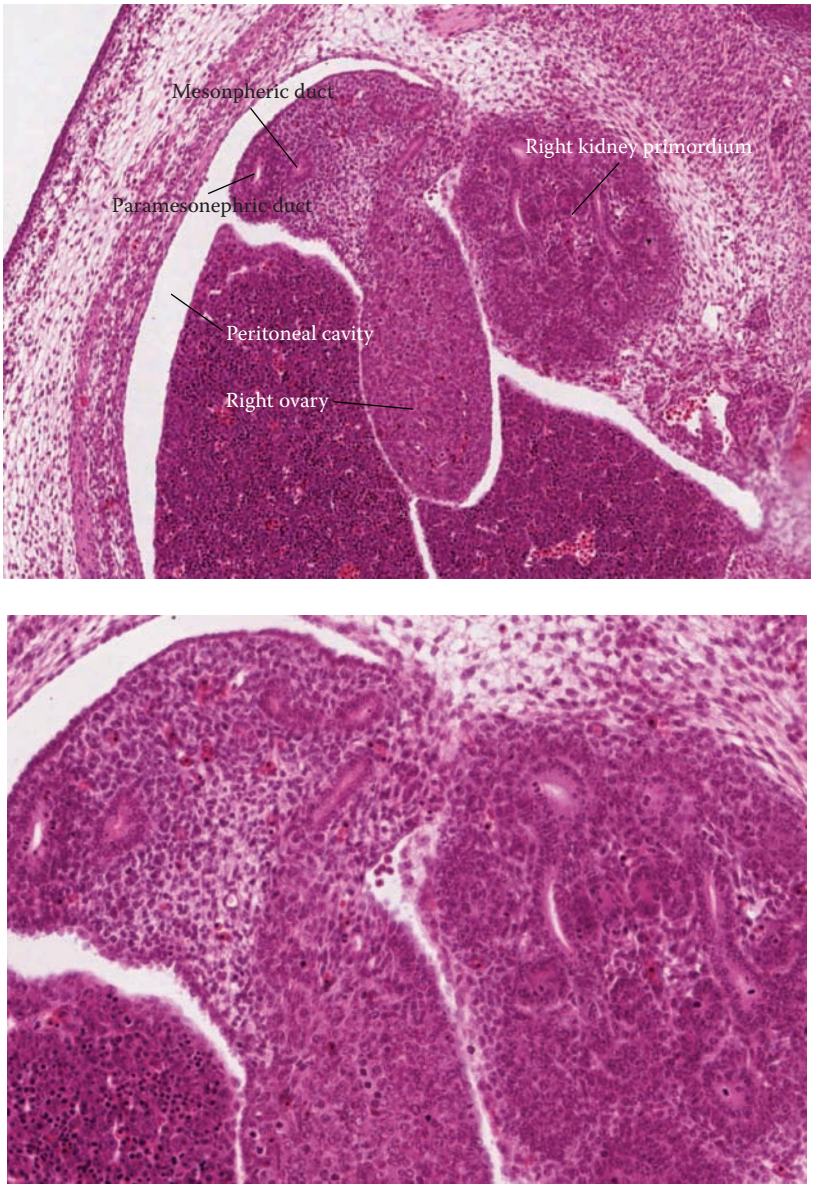

FIGURE 9.2 This is a medium (top) and high (bottom) magnification of Figure 9.1 to illustrate the right ovary. Its upper (posterior) pole is adherent to the urogenital ridge that contains both the paramesonephric and mesonephric ducts. The medial aspect of a considerable part of this ovary appears to be closely associated with the primordium of the right kidney, while most of the lateral margin and about half of its antero-medial margin appears to be closely associated with one of the lobes of the liver.

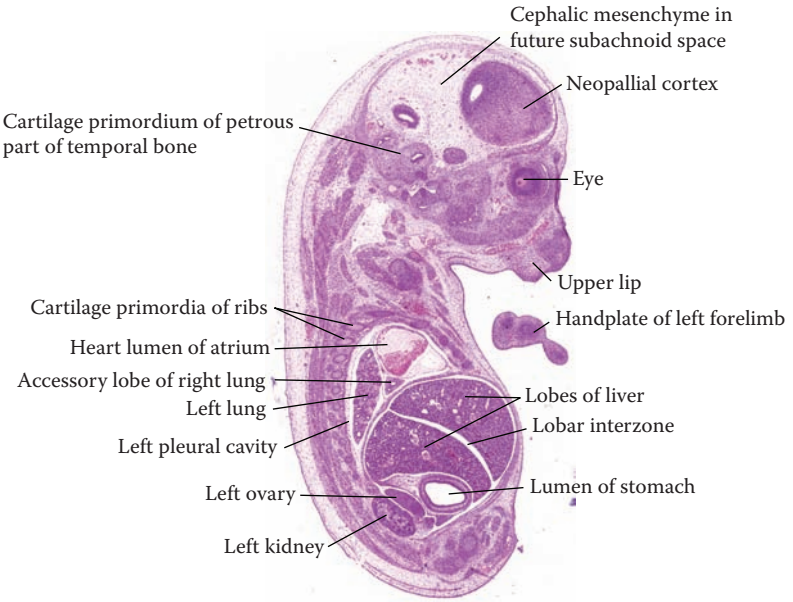

FIGURE 9.3 (E13.5_Fig9.3.svs) This represents a low magnification, sagittal section through much of the left side of the peritoneal cavity of a mouse embryo (E13.5–14) that is stained with H&E. The lumen of the stomach is readily recognized, and the left ovary is located between the posterior wall of the stomach and the anterior wall of the left kidney.

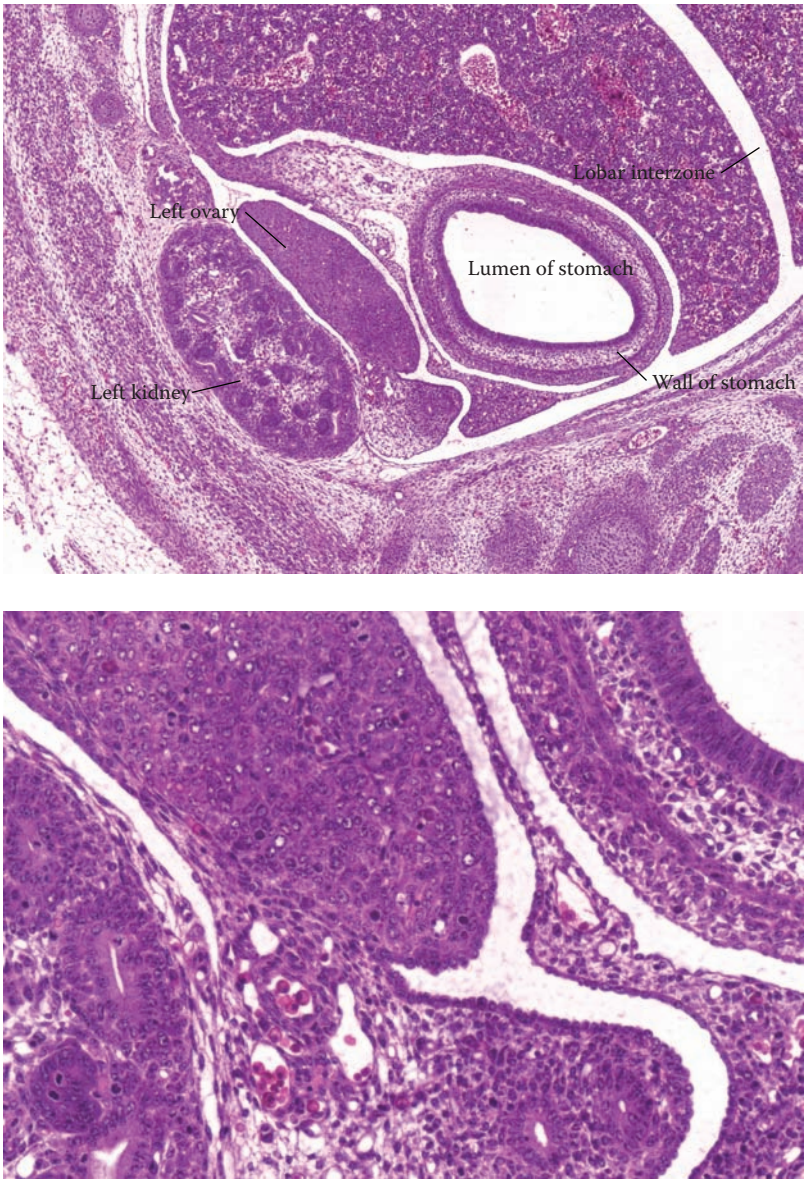

FIGURE 9.4 This is a medium (top) and high (bottom) magnification of Figure 9.3. The medium magnification image emphasizes the close relationship of the left ovary to the posterior wall of the stomach and the anterior surface of the left kidney, although the latter is clearly seen to be covered by a relatively thin layer of peritoneum. The inferior pole of the ovary is adherent to the posterior abdominal wall via the urogenital mesentery.

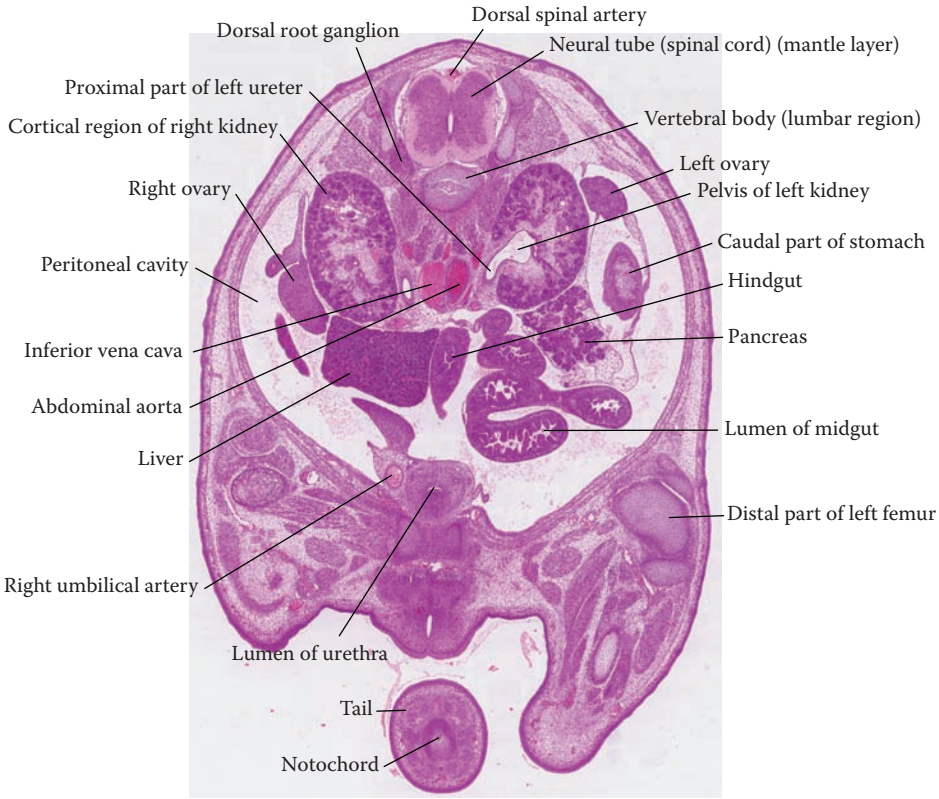

FIGURE 9.5 (E15.5_Fig9.5.svs) This represents a low magnification, transverse section through the mid to upper abdominal region of a mouse embryo (E15.5) that is stained with H&E. The right and left kidneys are now seen to be well differentiated, particularly in their cortical regions. A left ovarian bursa is also clearly seen, although there is no evidence of an ovarian bursa on the right side of this section. In the midline anteriorly, it is possible to recognize the base of the urinary bladder within which the lumen of the urethra is clearly seen. The right umbilical artery, a small region of the hindgut and midgut, is also readily recognized, as is the left renal pelvis and proximal parts of both the left and right ureters.

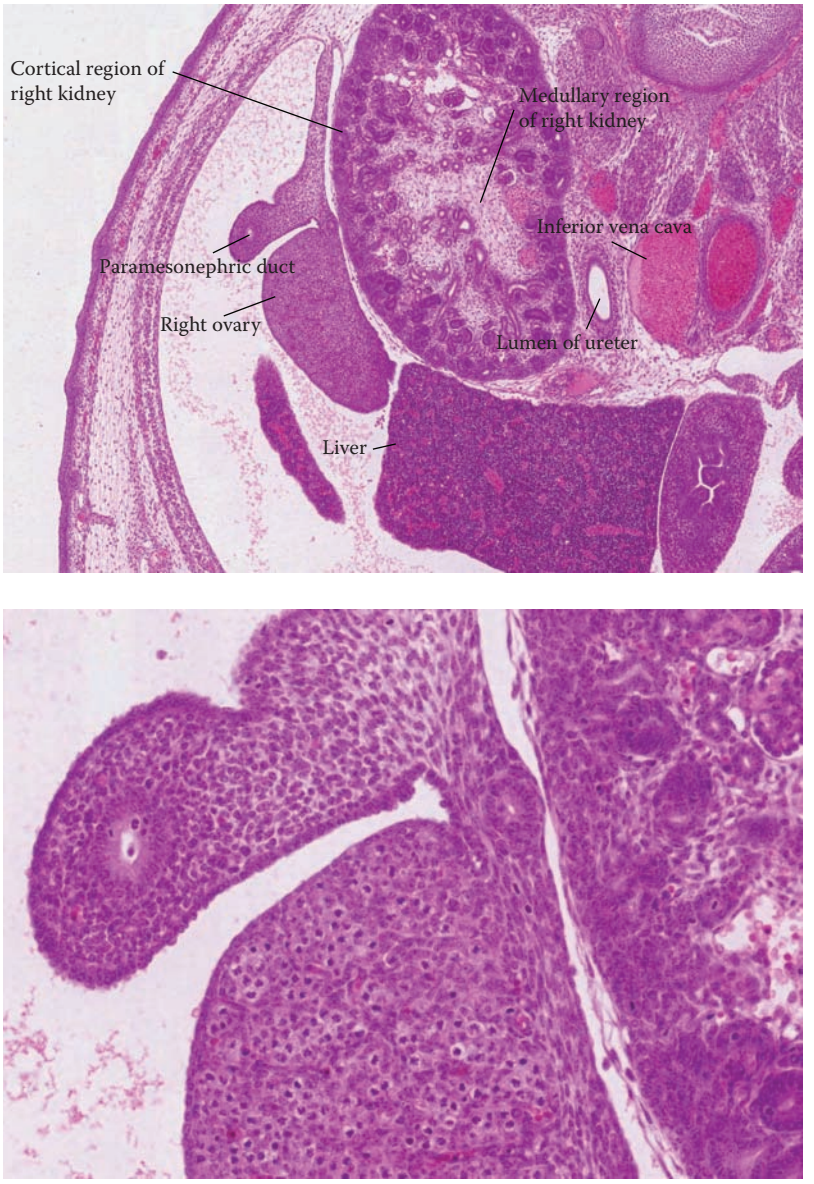

FIGURE 9.6 This is a medium (top) and high (bottom) magnification of Figure 9.5 demonstrating the right ovary, showing its close relationship to the right paramesonephric duct. The detailed histological morphology of the components of the cortical region of the right kidney is also now clearly seen.

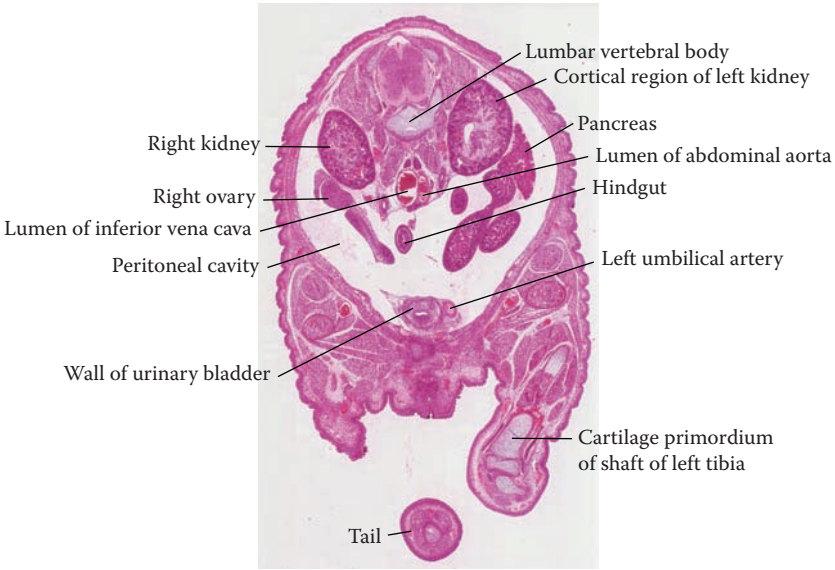

FIGURE 9.7 (E16.5_Fig9.7.svs) This represents a low magnification, transverse section through a similar region of the abdomen to that displayed in the previous sections of a mouse embryo (E16.5) that is stained with H&E. The right ovary is clearly seen, although the left ovary is not seen in this section. The wall of the proximal region of the urinary bladder is clearly seen, and this is closely associated with the left umbilical artery. The lumen of the abdominal aorta and the more dilated lumen of the inferior vena cava are also readily recognized.

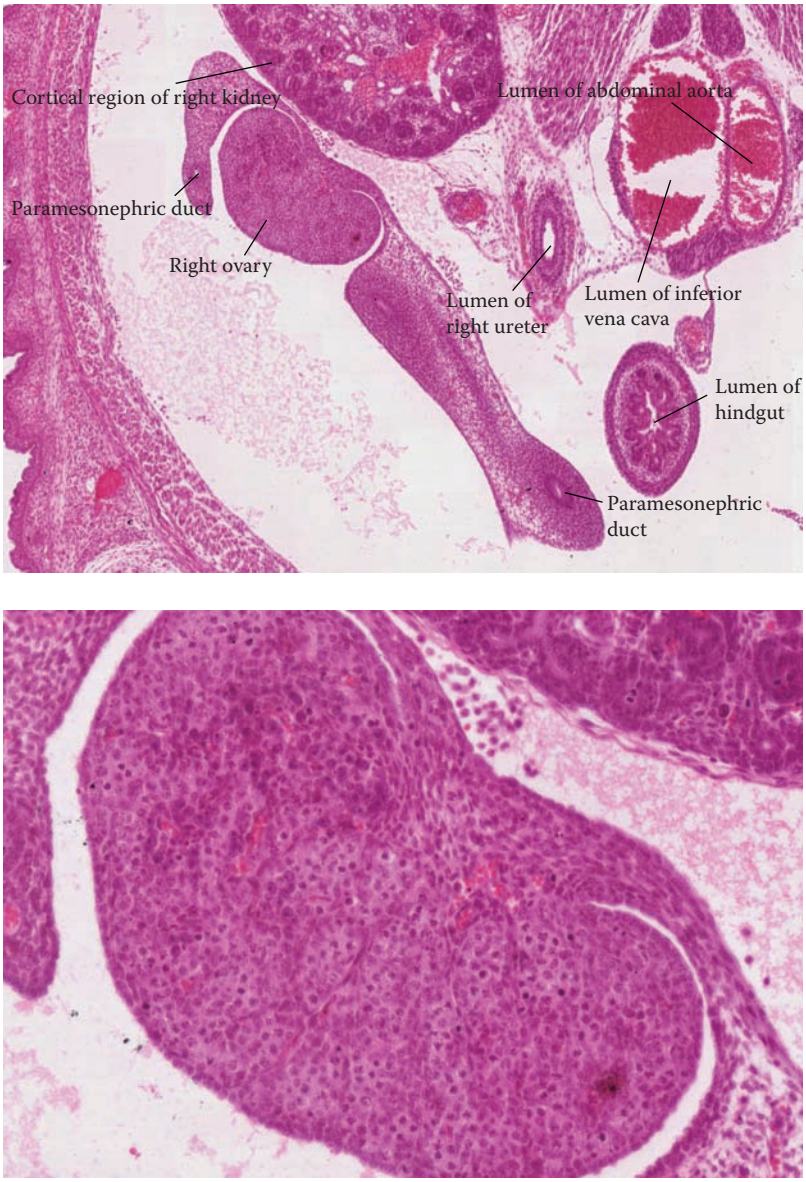

FIGURE 9.8 This is a medium (top) and high (bottom) magnification of Figure 9.7 showing the right ovary. Close to the dorso-lateral region of this ovary the paramesonephric duct is destined to form the oviduct, while more anteriorly and caudally this part of the paramesonephric duct will differentiate into the right uterine horn. The latter is closely associated with the transverse section through the hindgut region as it descends caudally toward the pelvis. The characteristic "dotted" histological morphology of the ovary is now also clearly seen in this section.

The Cortex

The outermost portion of the human cortex, as in the mouse, termed the *tunica albuginea,* develops as a distinct layer at about the time of birth and increases in width and fibrous density thereafter. In the mouse, this part is developed far less prominently. A single layer of squamous to cuboidal coelomic (or peritoneal) epithelium covers it. The latter layer was formerly termed the *germinal epithelium* because it was (erroneously) believed that this was where the germ cells originated. The cortical layer subjacent to the tunica albuginea is highly cellular. Within the innermost region of the cortex are located the oocytes, the derivatives of the primordial germ cells (PGCs). Even by the time of birth, a layer of follicular cells surrounds them to form the so-called primordial follicles. Within these, the oocytes are usually surrounded by often two or more layers of follicular cells. By birth, the oocyte usually measures about 25 μm in diameter. By the time of puberty, these follicles are embedded in stromal tissue, and one or occasionally a number of these follicles are destined to become ovulated during each menstrual cycle. Following ovulation, the follicular cells that remain in these so-called antral follicles give rise to the corpus luteum (or corpora lutea). In the mouse, the detailed morphology, as indicated above, is believed to be similar to that in the human, although no genes have yet been identified as having a specific role in ovary development.[20]

In humans, if pregnancy occurs, the corpora lutea are destined to form the corpora lutea of pregnancy. The follicular (or granulosa) cells that are not ovulated become transformed into progesterone-secreting cells, and these maintain the pregnancy during its first trimester (see below). Should pregnancy not occur then, after about 14 days, the corpus luteum regresses completely and is replaced by an avascular area of scar tissue termed the *corpus albicans*. In the human, the cortex and medulla merge into one another without a clear line of demarcation. This is unlike the situation in many other primates, such as the rhesus monkey, where a clear demarcation is observed between the cortical and medullary tissue of the ovary.[5]

Granulosa Cell Compartment

In addition to the PGC, the granulosa cells represent a critical component of the cortical region of the ovary. These surround the germ cells but are critically not in contact with the vasculature of the gonad.[21] The granulosa cells are connected to each other, however, by an extensive system of intercellular gap junctions so that they effectively form a functional syncytium. Their function is probably to facilitate transport of small molecules both between neighboring granulosa cells and into the oocyte through the zona pellucida. The latter is achieved via the cytoplasmic processes that penetrate through the zona pellucida and allow contact to be made with the surface of the oocyte. It is likely that this is also necessary for the control exerted by the follicular (or cumulus) granulosa cells on the resumption of meiosis.

Various classes of granulosa cells have been identified. These include the mural and antral granulosa cells. The mural granulosa cells abut the basement membrane of the follicle, and it has been suggested that these cells account for the majority of steroidogenesis that occurs in the follicle.[22,23] By contrast, the antral granulosa cells that immediately surround the oocyte in mature follicles appear to be both steroidogenically and metabolically less active than the mural granulosa cells.

The Interstitial Compartment

Various authorities[24] reported that they have recognized four classes of interstitial cells in the human fetal ovary: (1) primary interstitial cells; (2) theca-interstitial cells; (3) secondary interstitial cells; and (4) hilar interstitial cells. These cell types are recognized both by their function and location within the ovary. The first class of cells within this group is said to constitute a transient population of C19-steroid producing cells located in the medulla of the fetal ovary. They are first recognized at about 12 weeks of gestation and have completely disappeared by about 20 weeks. Their function

is unclear, but they closely resemble, morphologically, fetal testicular Leydig cells. They do not respond to gonadotropic stimulation, and it has been suggested that they may use circulatory steroidogenic precursors to produce androgens. The theca-interstitial cells are found in all developing follicles. They are located in the theca interna and constitute the stromal cells that lie close to the basal lamina around the granulosa cells and are also in close proximity to the theca externa (the stromal cells that constitute the outermost layer of the follicle). These cells form the principal C19-steroid-producing component of the follicle.

The secondary interstitial cells are said to represent hypertrophied theca interna remnants that survive follicular atresia. Despite their curious origin, they remain functionally and structurally unchanged. They are innervated by nonadrenergic neurons. The hilar interstitial cells are exclusively components of the ovarian hilum and morphologically closely resemble differentiated testicular Leydig cells. Nonmyelinated sympathetic nerve fibers innervate them. They are particularly prominent at the time of puberty, during pregnancy, and at menopause. Unlike the situation in the testis, the ovary does not constitute an immunologically privileged site. Thus, macrophages, lymphocytes, and polymorphonuclear granulocytes are observed at various stages during the ovarian life cycle.

The Ovarian Follicles

This is believed to be the most critical unit within the ovary both for germ cell differentiation and steroid production. These follicles are located within the ovarian cortical stroma and are subdivided into two types: the primordial follicles that do not grow and those that grow. While the majority belong to the former class, only a few will be recruited to the second class, and of these, only a small proportion is likely to be ovulated. Of those that display evidence of growth, those that develop as secondary or tertiary follicles are capable of developing in the absence of pituitary gonadotropic stimulation.[25] Only follicles to be ovulated are apparently recruited during the first few days of the cycle, during which they will be ovulated. It appears that the early phases of follicular growth occur in the preceding several cycles, which may take in the region of about 85 days, but its final differentiation into a Graafian follicle only occurs in the cycle during which it will be ovulated.[26,27]

While a considerable cohort of tertiary follicles, each measuring between 2 and 5 mm in diameter, are exposed to pituitary-derived follicle-stimulating hormone (FSH), if they fail to be stimulated then they are inevitably destined to undergo atresia. Of those follicles that respond to FSH stimulation, only one or occasionally a few so-called "dominant" or "mature" Graafian follicles[28] will be ovulated. It should be noted that the FSH these follicles respond to is produced during the luteal phase of the previous cycle and that the period of development of these follicles is in the region of 10–14 days. If the follicle is not selected for ovulation, then it undergoes the process of atresia during which both the oocyte and the majority of its associated granulosa cells die and are replaced by fibrous tissue. The thecal cells outside the basal lamina do not die but return to the pool of ovarian interstitial or stromal cells.[1] The cells that undergo atresia are all believed to undergo the process of apoptosis.[29] It has been suggested that the dominant follicle produces a *follicular regulatory protein* and that its principal effect, in the human, is to suppress the response of the other follicles to gonadotropic stimulation.

OVULATION: PRELIMINARY OBSERVATIONS

It was formerly believed that the tertiary follicles destined to mature to form Graafian follicles and then undergo ovulation had responded to pituitary-derived FSH. This allowed folliculogenesis to be completed. The mature Graafian follicles were then exposed to pituitary-derived luteinizing hormone (LH). This stimulated oocyte maturation to occur so that the so-called "resting-phase" oocytes were stimulated to progress from the dictyate stage of meiosis I, then enter and progress

through the first meiotic division and finally cease development and be ovulated at metaphase of the second meiotic division.

While this is the stage when ovulation occurs in the human and in many other mammals, this is not invariably the case. In the dog and fox,[30] for example, and in many other mammals, ovulation occurs when the oocyte is at metaphase of the *first* rather than the *second* meiotic division, as is the case noted previously. In these species fertilization acts as the normal event that stimulates the egg to complete the first and then the second meiotic division, with the extrusion of initially the first and then the second polar body. More unusual sites of fertilization have also been described, such as in the tenrecs. In these animals, the ripe follicles possess no antrum, and large numbers of spermatozoa penetrate the very thin ovarian surface epithelium and the theca overlying the follicle. They then enter the epithelial tissue of the secondary follicle, reach and pass through the zona pellucida, and then fertilize the egg. At the time of fertilization, the egg is a secondary oocyte, having already extruded its first polar body. Following fertilization, the fertilized egg is detached from the follicle and then passes into the ovarian bursa and then into the oviduct. In a few cases, accidental retention of the cumulus and oocyte occurs even in ruptured vesicular follicles in a small number of species. This may result in an "ovarian pregnancy," although this has so far been reported only in the two genera of tenrecs—the *Setifer* and *Hemicentetes*—described by Strauss.[31]

In the human as well as in the mouse, the egg is ovulated at metaphase of the second meiotic division, and fertilization normally occurs in the ampullary region of the oviduct. This then acts as the normal stimulus to induce the egg to extrude the second polar body following the completion of the second meiotic division. The expulsion of the oocyte is associated with the expulsion of its surrounding antral or cumulus cells and probably all of the associated follicular fluid.[32] By an unknown mechanism, but probably due to chemotaxis, the oocyte is usually directed through the fimbriated os and thence into the ampullary region of the oviduct to await the fertilizing spermatozoon. In the absence of an ovarian bursa, the unfertilized egg within its oocyte-cumulus complex would otherwise have been expelled into the peritoneal cavity. It is of importance to be aware that the ampullary region of the oviduct provides the appropriate environment for fertilization to occur. The fertilized egg then undergoes cleavage, and at the morula stage, shortly after compaction, it is transported through the utero-tubal junction into the uterus. Recent information suggests that this represents an oversimplification of the events that occur at the time of, and leading up to, ovulation.

MORE RECENT OBSERVATIONS

While folliculogenesis is clearly associated with the rapid enlargement of the follicle, its protrusion at the surface of the ovary, and the subsequent rupture of the follicle, the underlying factors involved in these events have recently been shown to be more complex than formerly thought. In the human, ovulation occurs about 34–36 hours after the start of the LH surge, and the site on the surface of the ovary where the Graafian follicle ruptures is now termed the *stigma* (or *macula pellucida*). This is said to be due to the local cessation of blood flow to the theca interna and consequent thinning of this region of the follicle from about 80 μm to about 20 μm or less.[33]

In this location, and through this site, the oocyte-cumulus complex is expelled into the peritoneal cavity. It has also now been demonstrated that stigma formation and subsequently the rupture of the stigma is associated with the production of proteases, such as collagenase. These act on local substrates in the basal lamina.[34] It has also been demonstrated that the levels of plasminogen activator increase within the follicle at this time with the associated conversion of plasminogen into plasmin.[35]

It appears that granulosa cells exclusively express the follicle-stimulating hormone receptor (FSHR). By contrast, the luteinizing hormone/choriogonadotrophin receptor (LHCGR) is expressed principally by the theca-interstitial cells of all follicles and by the granulosa cells of the Graafian follicles. Accordingly, the preantral follicles respond to the presence of FSH but not luteinizing hormone beta (LHB), while antral follicles are able to bind both FSH and LH.[36] It has therefore been hypothesized that LH receptors may appear on granulosa cells that have formerly been exposed to

FSH.[21] During ovulation, the principal role of FSH is the induction of follicular maturation, through the growth and differentiation of the follicle, and this view is consistent with the analysis of human females with mutations that disrupt the function of the follicle-stimulating hormone beta (FSH-β or FSHB) subunit gene. Such individuals closely resemble those with gonadal dysgenesis. They have poorly developed secondary sexual features and possess high levels of circulating FSH and LH. They do, however, show evidence of early follicular growth. This ovarian phenotype is therefore completely different from that observed in individuals with Turner's syndrome, as these possess streak gonads associated with an absence of growing follicles.[37]

Another important action of FSH is the induction of LHCGR in granulosa cells, and this action appears to be augmented by the presence of estrogens.[38] Once induced, the granulosa cell LHCGR induced by FSH appears to require the presence of FSH to maintain it. However, in the absence of LH, oocyte maturation and ovulation will not occur.[39]

ROLE OF LUTEINIZING HORMONE IN INDUCING OVARIAN FUNCTION

LH is required not only to allow the oocyte within the Graafian follicle to mature but also to guide the process of ovulation and subsequently the formation, maintenance, and functioning of the corpus luteum. LH also plays a series of other roles. Possibly most significantly, LH is now considered of importance in facilitating the developmental progression of small antral follicles to the preovulatory phase.[40,41] It is also believed to act on the theca-interstitial cells of the small follicles to promote the biosynthesis of C19-steroids.[42]

Ovarian Steroidogenesis

During the first half of the menstrual cycle in the human, the preovulatory follicle secretes estradiol, while the CL secretes both estradiol and progesterone during the second half of the cycle. The production of these two steroids occurs within the follicle and CL and is under the control of both LH and FSH. The release of these hormones is, in turn, controlled by the gonadotropin-releasing hormone 1 (GNRH1) produced by the hypothalamus. The feedback of hormones produced by the ovary (principally from the granulosa and theca cells) acts on the hypothalamus and thus regulates the release of the gonadotropic hormones. Analysis of venous blood from the ovaries has revealed that they secrete pregnenolone, progesterone, 17α-hydroxyprogesterone, dehydroepiandrosterone (DHEA), androstendione, testosterone, estrone, and estradiol.[43,44] However, such studies did not identify the cells involved in the production of these steroids. Other studies revealed that progesterone and 17α-hydroxyprogesterone were the principal products of the CL.

It appears that three major classes of ovarian steroids are produced: C18-steroids; C21-steroids; and C19-steroids. One of the principal C18-steroids is estradiol. This regulates gonadotrophin secretion and promotes the development of the secondary sexual features, growth of the uterus, thickening of the vaginal mucosa, thinning of the cervical mucus, and growth of the ductal system in the breast. The principal C21-steroids are the progestogens and include pregnenolone (the precursor of all ovarian steroid hormones), progesterone, and 17α-hydroxyprogesterone (believed to have little if any biological activity). Progesterone is the principal secretory product of the CL and is required for implantation of the fertilized egg, and the maintenance of pregnancy for the first 2–3 months, after which the hormones produced by the placenta take over this role.

The C19-steroids include DHEA, androstenedione, and testosterone and are produced by the thecal cells and, to a lesser extent, by the ovarian stroma. Some of the androstenedione is secreted into the plasma, while the rest is converted into estrogen by the granulosa cells. It appears that only testosterone and dihydrotestosterone (DHT) are true androgens and are capable of reacting with the androgen receptors (ARs). All steroids produced by the ovary are derived from cholesterol, and steroidogenesis occurs following the movement of cholesterol into the appropriately located ovarian mitochondria.

Corpus Luteal (CL) Formation and Functioning

Following ovulation and the associated release of the follicular fluid from within the Graafian follicle, the elevated level of LH initiates the transformation of granulosa cells of the oocyte-depleted follicle into lutein cells. The basal lamina that previously separated the granulosa cells from the theca interna breaks down. This may also be associated with some loss of blood into the thecal region. Shortly afterward, the granulosa and theca interna cells hypertrophy and at about the same time begin to accumulate lipid droplets. They are then converted into the characteristic lutein cells so that the oocyte-depleted Graafian follicle is converted into the CL. At about this time, the capillaries of the theca interna become disseminated around the granulosa cells, and the fibroblast cells that form a connective tissue matrix in this location accompany these blood vessels.

Two types of lutein cells are recognized. Cells in the interior of the CL that develop from the granulosa cells constitute the majority of the cells of the CL and are then termed the *granulosa lutein cells*.[45] The smaller cells located at the periphery of the CL are termed the *theca lutein cells*. These two cell types are believed to respond differently to the presence of LH. It should also be noted, however, that the theca lutein cells are found in the CL only of a relatively small selection of mammalian species.

The principal secretory product of the CL is progesterone, although in the human and in other primates estradiol and other steroids are also secreted. The diameter of the individual granulosa lutein cells increases from about 30 μm up to about 50 μm in diameter if pregnancy occurs, when the CL becomes converted into the CL of pregnancy. While the mitochondria tend to be prominent, they are also variable in shape in the early CL. If pregnancy supervenes, then the mitochondria in the granulosa lutein cells tend to become larger and spherical in shape. The cytoplasm of these cells also tends to possess an abundance of principally smooth endoplasmic reticulum, numerous lipid droplets, and large numbers of small Golgi complexes. While the theca lutein cells display many of the ultrastructural features of the granulosa cells, the large spherical mitochondria are not seen in these cells. The Golgi complexes, however, tend to be larger and fewer in number.[46,47] These cytological differences are believed to be due to the different functions of these cells, with the theca lutein cells secreting estradiol and estrone in addition to progesterone.

If fertilization fails to occur, then the CL collapses and regresses, the lutein cells undergo autolysis, and the constituent cells become invaded by macrophages. As a consequence, the CL becomes converted into the *corpus albicans*. As indicated previously, while the majority of the oocytes and their associated follicles are destined to undergo atresia, the process is initiated during the second half of fetal life of the individual. The rate of oocyte loss indeed increases from birth to puberty and, to a lesser degree, throughout the female's reproductive life, until the ovaries are completely depleted of their oocytes and follicles at menopause. Comparable information is available on the histological morphology of the CL of pregnancy in the rat and in a number of other species.[48,49]

It is relevant to note here that while gestation in the mouse usually lasts for 19–20 days, the ovaries are believed to be essential for the maintenance of pregnancy until 1 or 2 days before parturition.[49] Like the situation in the rat, it appears that the pituitary is required only for the first 11–12 days of pregnancy. The CL of pregnancy increases in size on or about the eighth day after mating, attains its maximum growth by about E13, and does not accumulate cholesterol until about 2 days before parturition.[49,50]

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10 The Testis

INTRODUCTION

While the testes, like the ovaries, are paired and homologous structures, both of which play a critical role in germ cell differentiation and in the maintenance of the reproductive systems of the male and female, respectively, at the gross morphological level they appear to have relatively little in common. As has been noted previously, both develop from the genital (or gonadal) ridges on the antero-medial aspect of the mesonephros and at an early stage become invaded by the diploid primordial germ cells (PGCs). If PGCs possess an XX sex chromosome constitution, then the "indifferent" gonads are destined to develop into ovaries, while if PGCs possess an XY sex chromosome constitution, then the gonads are destined to develop as testes. In this case, the presence of the Y chromosome was the male sex-determining factor.[1–4] It was formerly believed that the male sex-determining activity of the Y chromosome was due to the presence of a gene or genes termed *testis-determining factor* (*TDF*) in humans and *Tdy* in mice. It is now believed that the *SRY* gene in the human (and the *Sry* gene in the mouse) is essential for testis determination (see below).[5]

Similarly, the internal genital duct system and external genitalia develop appropriately, either in the direction of femaleness or maleness, respectively. Both gonads are relatively easily divided into an outer cortical and an inner medullary region. However, while oogenesis occurs within the cortical region of the ovaries, spermatogenesis occurs within the seminiferous tubules located within the medullary region of the testes. In various other regards, both at the gross morphological and at the histological level, the gonads appear to have little in common.

The testis represents the most critical component of the male reproductive tract. While the *Leydig* (or interstitial) cells play a critical role in its endocrine activity, these exclusively reside in the interstitial region between its seminiferous tubules.[6] These cells synthesize androgenic steroids, of which *testosterone* is particularly important because it is essential for maintaining spermatogenesis and promoting the development of the Wolffian duct system into the male reproductive tract.[7] This hormone is also released into the circulation and is critical for the maintenance of the male secondary sexual characteristics as well as the functioning of the various accessory glands of the reproductive tract that play a critical role during reproduction. These glands include the seminal vesicles, the prostate, and the bulbo-urethral glands. Other hormones and hormone-like substances are also produced elsewhere in the testis, such as by the *Sertoli* (or sustentacular) cells, and the various roles played by these substances, such as *anti-Müllerian Hormone* (AMH; also known as Müllerian-inhibiting substance, or MIS), is discussed later in this chapter.

As the early developmental events associated with the conversion of the so-called "indifferent" gonads and reproductive ducts have been considered in detail elsewhere in this volume (see Chapter 8) these are not considered here. Similarly, as the events observed up to the time of birth and shortly afterward in the human and in the mouse have also been considered, these are only briefly alluded to here. In the present chapter, unlike the majority of the other chapters, attention is drawn particularly to the events that occur during the postnatal period and up to and including puberty. The latter is clearly associated with the events associated with the onset of sexual activity; the various roles of the testis in the adult are also briefly considered here.

For histological observations on the prenatal development of the mouse testis see Figures 10.1 through 10.6.

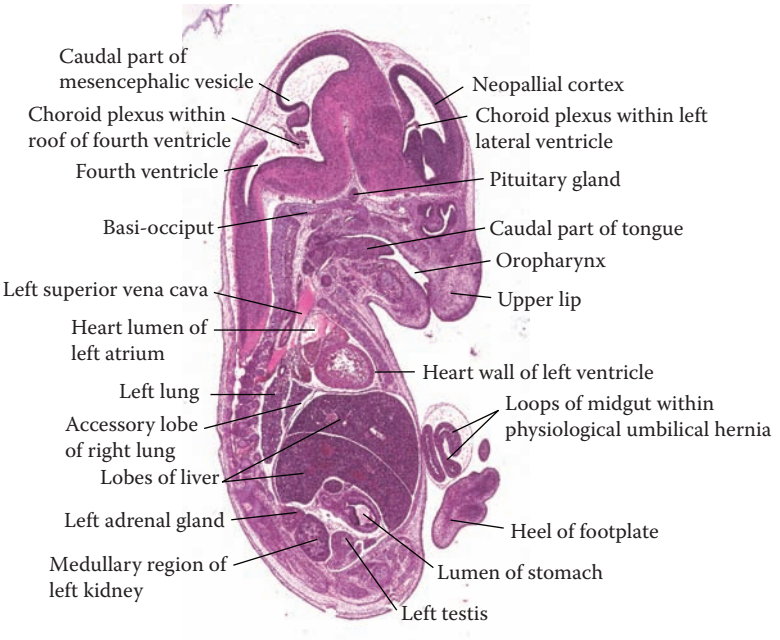

FIGURE 10.1 (E14.5_Fig10.1.svs) This represents a low magnification, sagittal section through the left side of the abdominal region, close to the median plane. The pyloric region of the stomach is also seen in this section of a mouse embryo (E14.5) that is stained with H&E. The posterior surface of the left testis is located close to the anterior cortical surface of the left kidney. The location of the left adrenal gland is also clearly seen in this section.

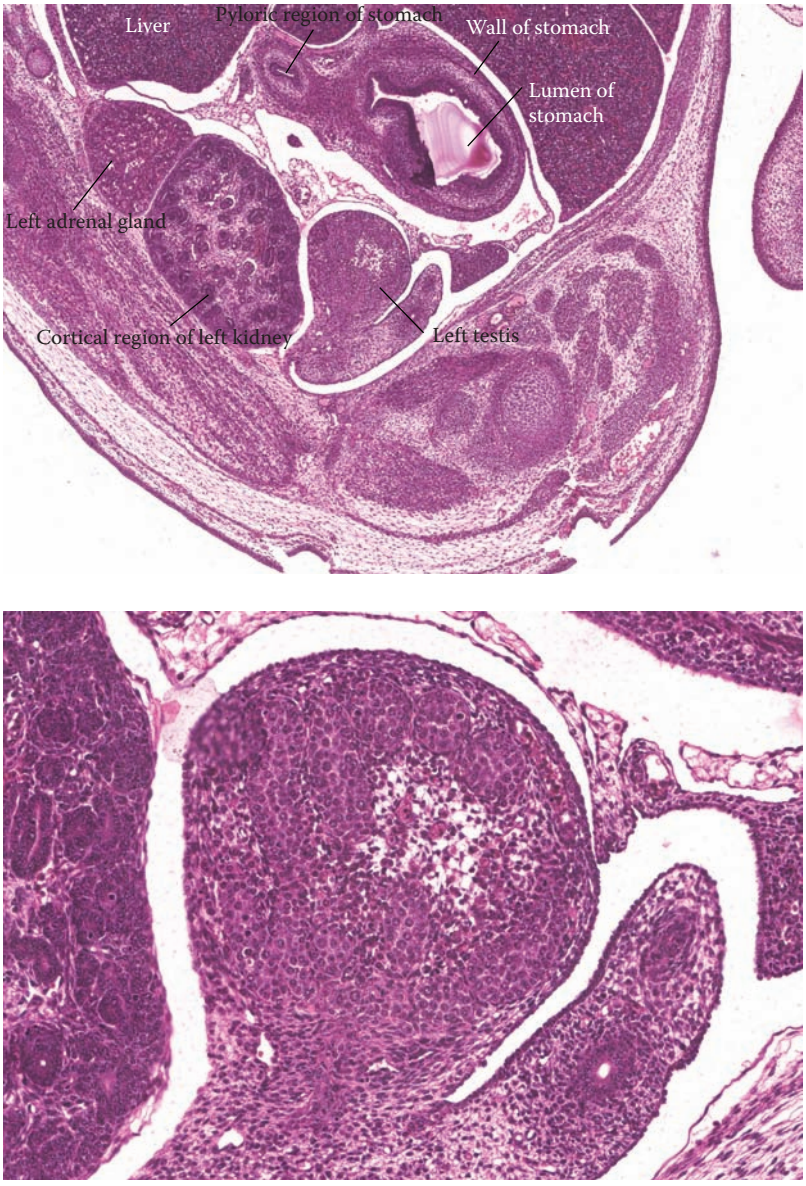

FIGURE 10.2 This is a medium (top) and high (bottom) magnification of Figure 10.1 showing the left testis and its immediately surrounding structures. It is just possible to see early evidence of the medullary region of this testis with what are now recognizable as the seminiferous tubules. These also appear to be closer to the outer (cortical) region of this gland, although they develop within its central (medullary) region. Note the early evidence of the glomerular structures developing in the cortical region of the left kidney. It is also just possible to make out the mesonephric duct that will shortly differentiate to form the ductus (or vas) deferens.

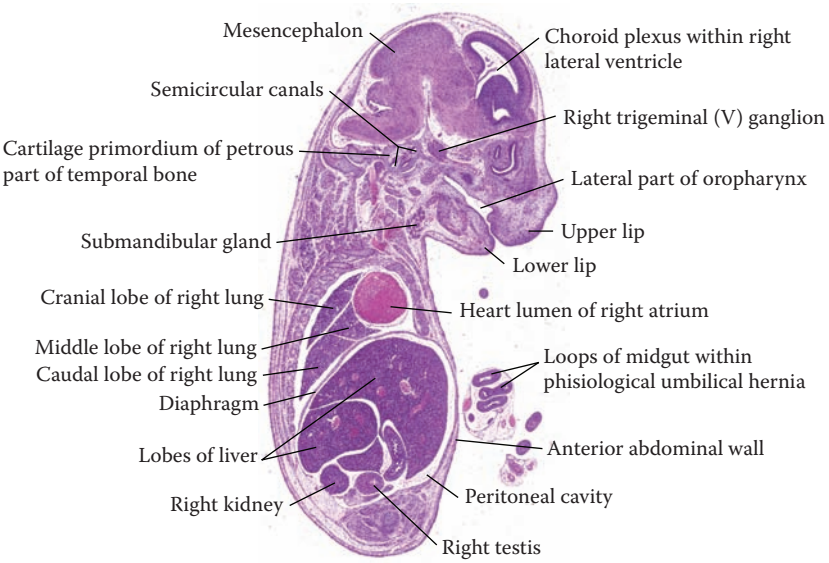

FIGURE 10.3 (E15.5_Fig10.3.svs) This represents a low magnification, sagittal section through the left side of the abdominal region of a mouse embryo (E15.5–16) that is stained with H&E. Despite its low magnification, the characteristic morphological appearance of the testis is now evident in this section. The restricted space available in the peritoneal cavity is also seen here and emphasizes the limited room available in this region for the midgut that is now almost exclusively present within the physiological umbilical hernia.

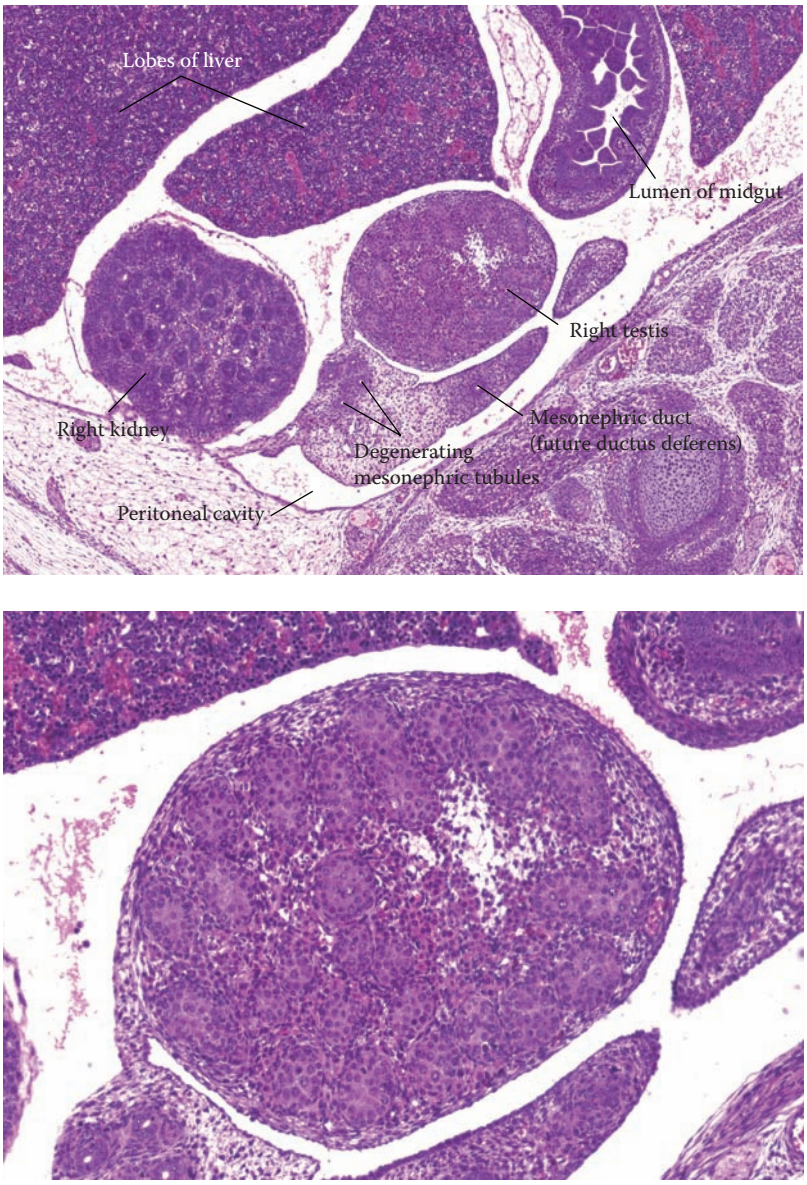

FIGURE 10.4 This is a medium (top) and high (bottom) magnification of Figure 10.3 illustrating the right testis. Particularly clearly seen in this figure are the degenerating mesonephric tubules located close to the rostral region of this testis and the proximal region of the mesonephric duct destined to form the rostral part of the ductus (or vas) deferens.

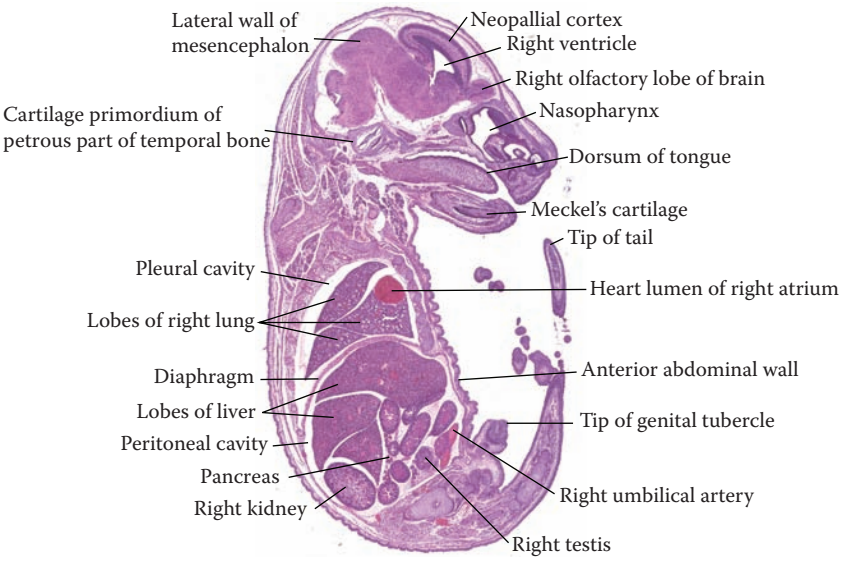

FIGURE 10.5 (E17.5_Fig10.5.svs) This represents a low magnification, sagittal section through the right side of the abdominal region of a mouse embryo (E17.5) that is stained with H&E. The characteristic histological morphological appearance of the testis is seen in this section. With the disappearance of the physiological umbilical hernia, there is now an increased volume in the peritoneal cavity to accommodate the midgut.

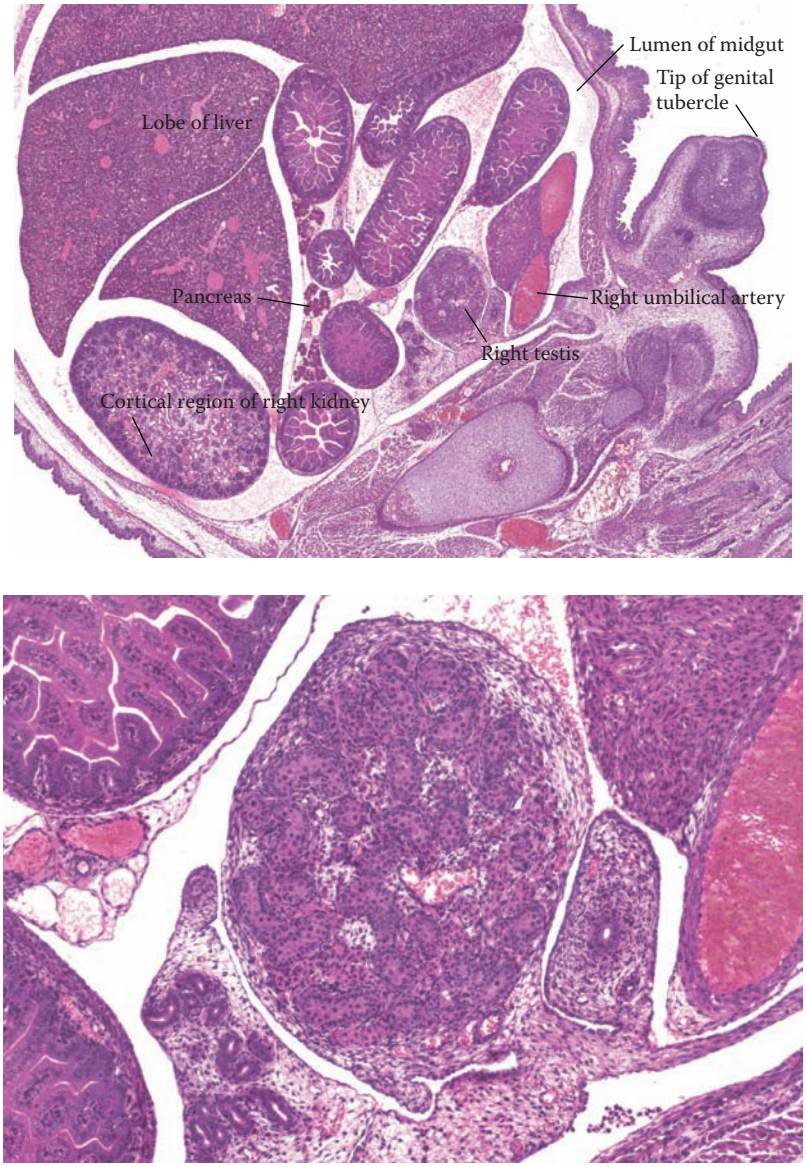

FIGURE 10.6 This is a medium (top) and high (bottom) magnification of Figure 10.5 illustrating the caudal part of the abdominal cavity. Later in gestation, both testes will descend slightly and will be accommodated in the pelvic part of the peritoneal cavity. In addition to the presence of the degenerating mesonephric tubules, it is just possible to recognize a transverse section through the mesonephric duct, which appears to be relatively closely related to the right umbilical artery in this section. The ducts' presence clearly indicates that this section is some distance from the midline, because there is evidence neither of the wall nor lumen of the urinary bladder in this section. It should also be noted that a well-differentiated genital tubercle is visible here.

GROSS VARIATIONS IN THE MORPHOLOGICAL FEATURES OF THE TESTES AND POSSIBLE CAUSES OF REDUCED FERTILITY IN THE HUMAN MALE

The overall size of the testes in the adult human and in other primates is extremely variable. While their overall shape is similar between the different species, being ovoid in form and, in the human, about 4–5 cm in length, 2.5 cm in width, and 3 cm in antero-posterior diameter, even within different racial groups the average weight of the testes varies considerably. Thus, in Scandinavian Caucasians they weigh about 21 g, while in Chinese their weight is closer to 8.8 g. In other primates, there appears to be little correlation between testicular weight and overall body weight. For example, in the chimpanzee, which weighs about a quarter of the weight of a gorilla, the testes are four times heavier. Similarly, according to Fawcett,[8] while the daily production of spermatozoa in the human is about 94.6×10^6 per testis, or about 5.6×10^6 per gram of testicular tissue, the number produced by other species is far higher. This source indicates that it may range from about 20×10^6 per gram of testis in the rat to 23×10^6 per gram of testis in the boar. However, taking into account the weight of the two testes in the boar, this suggests that the daily production of spermatozoa may be as high as 16.2×10^9.

Over many years, profound agitation has been expressed by experts in the field of human male fertility regarding what appears to be a real decline in the daily production of spermatozoa and an associated decrease in male fertility. A number of suggestions have been made to account for this, of which one of the more commonly suggested causes would appear to be the presence of increasing levels of environmental toxins.

Many factors have a profound influence on sperm count and fertility, and an extreme variability is observed between individuals and between ejaculates. Some of the factors involved include Sertoli cell number (see later),[9,10] ejaculatory frequency, season, scrotal heating (i.e., the time spent seated, see below), and aging. Evidence regarding the possible role of exposure to pesticides and other environmental toxins, however, while commonly believed to play a critical role, has yet to be established and has certainly not been confirmed. However, concern has been expressed about the accumulation of lipid-soluble pesticides in fat. What is indisputable, however, is the increase in the incidence of testicular cancer in Western countries over the past 50–100 years, and this alone strongly suggests that something in our environment and/or lifestyle is having a profound effect on early male reproductive development. For an overview of the topic of influence of environmental factors on male fertility see Sharpe.[11]

It is relevant to note here that Harshbarger and colleagues studied the influence of herbicides from the blueberry fields in the United States, as they appear to be capable of inducing testicular cancer in clams. They were later able to correlate their invertebrate findings to the induction of similar tumors in humans in the same geographic regions.[12–18]

It has been suggested that the optimum temperature for human spermatogenesis is 35°C and that mechanisms such as vascular countercurrent exchange in the *funiculus spermaticus* and selective contraction of the scrotal wall allows regulation of the scrotal temperature.[19–21] It had previously been reported that induced scrotal hyperthermia also had an impairing effect on spermatogenesis.[22] Similarly, artificial cryptorchidism also caused a significant decrease in sperm concentration and motility.[23] When this condition had been induced experimentally in the pig, spermatogenesis soon ceased,[24] although this was reversible if the intra-abdominal testes were exposed to a cooling device.[25] It is, however, important to appreciate that this procedure is going to be successful only if the spermatogenic cells are preserved in the abdominal testes. This procedure is often successful in sexually mature boars up to about 9 months of age, when the undescended testes are still capable of developing all cell types present in normally differentiated spermatogenic epithelium.[26] After this age, however, the outcome of this procedure is far less predictable, and fertility is a less likely outcome. In the adult human, the operation of scrotal orchipexy is recommended, but the success rate is somewhat variable.[27]

Another factor that is known to be of critical importance in relation to seminal quality and male infertility is sperm motility. Abnormal motility (*asthenospermia*) as an isolated disorder is

relatively commonly encountered, being observed in as many as 24% of cases of male subfertility. Asthenospermia is defined as sperm motility of less than 50–60%, or a decrease in linear, progressive movement characteristics. While individuals with this condition usually have associated defects in sperm production and morphology, isolated asthenospermia is observed in as many as 20+% of subfertile men.[28]

In a high proportion of subfertile men with asthenospermia concomitant abnormalities of sperm production and morphology are observed, although the precise cellular mechanism underlying oligospermia is largely unknown. However, the factors that induce impaired sperm production probably also affect structural and regulatory processes in the sperm tail that are necessary for normal movement, and these may be relatively easily demonstrated at the light microscopic level.[29] In attempting to treat this condition, it is important to be aware that when no specific cause is found, empiric therapy is unlikely to succeed. However, if a full diagnostic workup is carried out in these patients, it would at least be possible to avoid therapies that are unlikely to result in clinical pregnancy. Of the specific therapies available, none reverse the abnormalities observed in patients with *immotile cilia syndrome*, although in vitro fertilization (IVF) technology combined with zona drilling or intracytoplasmic sperm injection (ICSI) may have potential value in the treatment of this condition. If antisperm antibodies are found to be the cause, then immunosuppressive medication may be used in an attempt to reduce the antibody titers, although the results to date are not very impressive. If infection is the cause, then the individual should be treated with appropriate antibiotics. If the condition is unexplained, then, as indicated previously, therapeutic success is uncommon.[30,31]

Of couples seeking fertility treatment, 30 to 40% are diagnosed with male factor infertility. Diagnosis of the specific type relies on the results of semen analysis undertaken on a number of occasions, and in at least 60% of these cases it may not be possible to establish the etiology responsible. However, with the recent advances in molecular biology and molecular genetics it is now possible to establish the underlying cause in cases previously considered to be of idiopathic origin. In a proportion of the latter, the techniques of IVF and ICSI offer the possibility of reproductive success to a proportion of those previously considered irreversibly sterile. In cases where a genetic disorder is recognized, there is the increased risk of transmitting the genetic defects to future generations. For a detailed analysis of the various types of genetic disorders that may be associated with male infertility and other conditions known to be associated with chromosomal aberrations—such as Klinefelter's syndrome (85% XXY, 15% 47 XXY/46 XY); 47 XYY syndrome; Noonan's syndrome (usually 46 Xo/XY); Prader-Willi syndrome; Y-chromosome deletions; 46 XX males; and Down's syndrome (while females with this condition may become pregnant, males with this condition are almost exclusively sterile due to spermatogenic arrest)—the reader is advised to consult reviews on this topic.[32,33]

GONADAL SIZE IN RODENTS

For the sake of comparison, it is relevant to note that the dimensions and weights of the testes in the adult mouse and rat are as follows. In the mature mouse, the testis measures about 8.5 mm in length and about 5 mm in diameter and weighs about 85 mg.[34] In the mature rat, the testis measures about 20 mm in length and 14 mm in diameter and weighs between 2.0 and 3.5 g.[35]

DESCENT OF THE TESTES

This usually occurs in the human infant at or very shortly after the time of birth, with the descent of the testes into the scrotum. The fibro-muscular band (the *gubernaculum testis*) guides their descent, and probably also draws down a peritoneal diverticulum (the *tunica vaginalis testis*) closely associated with their anterior and lateral surfaces. Under normal circumstances, the narrow cleft that initially connects the region around the testis with the peritoneal cavity seals off so that the original connection disappears. Should, however, this connection remain, this potentially forms the route of

descent of an indirect (or congenital) type of inguinal hernia. The peritoneal sac that surrounds the testis is bilayered, with an inner *visceral* component (that is directly adherent to the outer surface of the testis) and an outer *parietal* component. A potential space or cleft that usually contains a small volume of clear serous-like (peritoneal) fluid separates these two layers. Under certain circumstances, this may fill with a substantial volume of peritoneal fluid to form a *hydrocele* of the testis.

In the human, descent of the testis is an essential event, and it is believed that a temperature several degrees lower than core body temperature is essential for normal spermatogenesis to occur. Should one or both of the testes fail to descend completely into the scrotum at the normal time, they may be vulnerable to trauma. This is particularly likely to occur if one or both of the testes are located somewhere along their normal path of descent, usually within the inguinal canal. Alternatively, the testis may descend and become located in the superficial inguinal pouch. Should a testis fail to descend into the inguinal canal and remain within the peritoneal cavity, a condition termed unilateral cryptorchism or cryptorchidism, then interference with spermatogenesis on the affected side almost invariably occurs. In a finite proportion of these cases, this condition is associated with malignant changes in the germ cells, leading to teratocarcinoma formation. Occasionally, in the human, unilateral failure of descent of a testis may be associated with congenital absence of a kidney (i.e., congenital *renal agenesis*) on the same side.

The situation in the mouse, and in many other species, is also different from that described above. This particularly applies to the anatomical relations of the testes in the adult. While descent of the testes into the scrotum occurs in the mouse, the scrotal extension of the peritoneum remains in communication with the peritoneal cavity through the inguinal canals. These communications (the *processus vaginalis*) remain patent throughout life, and, except during sexual activity, the testes are often in the retracted position. While for much of the time the testes in the mouse are retracted into the inguinal region, they are nevertheless at a slightly lower temperature than core body temperature. In the rat, it has been suggested that the voluminous fat body associated with the spermatic cord may prevent complete herniation of the testis into the peritoneal cavity. Similarly, it has been suggested that the fat pad may also play a part in reducing heat conduction from the body to the slightly cooler testis/epididymis complex. Bedford[36] suggested that testicular descent into the scrotum was not because the testes required thermo-regulation but because of the need for migration of the cauda epididymis to this cooler location. Accordingly, testicular descent was merely a mechanical event that enabled the cauda epididymis to achieve a lower than core body temperature. Bedford further argued that in crytorchid species (e.g., many insectivores, hystricomorph rodents) the cauda generally precedes the testis within the cremasteric sac and thus ensures that the epididymis occupies the coolest site there also. Other evidence based principally on comparative anatomical and from experimental studies was presented by Bedford to confirm this hypothesis. He also argued that the scrotal state may be linked to the sexual capacity of the male, in particular to the ability to produce fertile ejaculates repeatedly within a limited period of time. More recent experimental studies indicate that this may not in fact be the case, particularly in the light of studies on the effects of temperature on spermatogenesis.

Very occasionally in the human, the testes may be (pathologically) retractile in a similar way to what is normal in many other species. Clearly, in the human, this may result in an abnormal elevation of testicular temperature, which appears to have a dramatically detrimental effect on spermatogenesis. The presence of a varicocele may also have the same detrimental effect on spermatogenesis (see below) because of the increased temperature to which the testes are exposed. The surgical repair of this condition may be undertaken by either the subinguinal[37] or laparoscopic[38] approach. Complications are often encountered after both of these approaches, the most common reason for this being hydrocele formation, which is usually attributable to lymphatic obstruction. Its effect is uncertain, and this may lead to an elevated intratesticular temperature and an adverse effect on fertility. Extreme care during surgical intervention to preserve the lymphatics may significantly help to reduce the incidence of this complication.[39]

GERM CELL DIFFERENTIATION WITHIN THE TESTIS

As in the case of the ovary, it is essential that the germ cells are located within their appropriate compartment. In the female, the follicle represents the critical germ cell compartment. This contains the diplotene oocyte and granulosa cells, which are surrounded by a basement membrane. The germ cells in the male need to be located *within the testicular cords* (or seminiferous tubules). Similarly, it is essential that the steroid-producing Leydig cells develop *outside* the seminiferous tubules, within the interstitial compartment. As has been noted elsewhere (see Chapter 8), it is essential that the germ cells (at this stage these are termed the *prespermatogonia*) develop in close association with the presumptive Sertoli cells to facilitate initially spermatogenesis and subsequently spermiogenesis. However, it should be noted that in the male the germ cells do not enter meiosis until puberty. This contrasts with the situation in the female, where meiosis always precedes the formation of the follicle.[40] Furthermore, it should also be noted that if the male germ cells are not enclosed within the testicular cords, they invariably degenerate, although this may not be the case when, occasionally, PGCs migrate into ectopic sites and might then give rise to teratocarcinomas (see below).

In most species, virtually no mitotic activity is seen in the prespermatogonia from the period shortly after testicular differentiation is first achieved until the time of, or shortly before, puberty. Thus, in the rat, shortly before birth the prespermatogonia enter the so-called "cap phase." These cells are then arrested in interphase and contain the diploid amount of DNA.[41] In most species, this is the situation observed until shortly before puberty. By contrast, in the mouse, a different situation is observed, as mitotic activity is resumed shortly after birth.[42]

TESTICULAR FUNCTION DURING THE POSTNATAL PERIOD

Since most of the information available concerns the postnatal development of the testes in the human, this topic is considered first, which will provide the necessary baseline information on the events that occur during comparable periods of development in rodents. As any detailed consideration of the male accessory sex glands is beyond the scope of the present chapter, these are only briefly mentioned because of the complementary and critical roles they play in the production of the ejaculate. Because of slight species differences, but particularly the presence of certain structures in the mouse (e.g., the coagulating gland) that are not present in the human or do not function in the rat as they clearly do in the mouse, attention is given to these structures where appropriate.

TESTICULAR MORPHOLOGY AND FUNCTION DURING THE POSTNATAL PERIOD IN THE HUMAN

A very considerable volume of literature is available on the gross morphological features of the testis in the human adult.[8] As noted previously, it is essential for testicular function that the male gonad is subdivided into two distinct compartments. The testicular cords, or seminiferous tubules, are principally associated with spermatogenesis; the cellular tissue between these (within the interstitial compartment) has numerous other functions. One of the most important functions of the latter is played by the Leydig cells (see below) involved, among other functions, in the production of the androgenic steroids.

At the gross morphological level, the human testis is enclosed in a thick fibrous capsule (the *tunica albuginea*), and dense connective tissue septa extend inward to form the *mediastinum testis*. In the adult, the testicular cord component of the adult testis is subdivided into about 250 compartments or lobules. Each of the latter contains from one to four seminiferous tubules, each measuring about 150–250 μm in diameter. At the median apices of each of the lobules, the very convoluted seminiferous tubules straighten out and converge to form the *tubuli recti*. This occurs within the mediastinum where they enter and form the *rete testis*. Here they unite to form the efferent duct system that then connects with the epididymis. This effectively acts as the "drainage" system for

the seminiferous tubules, through which the morphologically "mature" spermatozoa then pass to the ductus deferens.

The tubular fluid that passes with the spermatozoa is then supplemented with secretions from the various accessory sex organs. In the human, and in other primates, unlike the situation observed in rodents (see below), none of the cells that line the tubules appear to resemble the myoid (i.e., muscle-like) cells observed in rodents, and consequently no evidence of contractility of the seminiferous tubules has been observed in these species.

Within the seminiferous tubules are substantial numbers of Sertoli (or sustentacular) cells, and they *support* the development of successive stages of the spermatogenic cell lineage from the primitive spermatogonia to the mature spermatozoa. The latter are then released into the lumen of the seminiferous tubules and pass with the associated luminal fluid toward the rete testis, after which they are released into, for example, the efferent ducts and the epididymis (see above). During much of spermatogenesis, principally from the spermatogonial stage, large numbers of germ cell progeny develop synchronously as a result of the presence of intercellular cytoplasmic *bridges*. As a result of their presence, the germ cells are for most of this period of differentiation joined and consequently behave as a syncytium. These cytoplasmic connections are severed only when the mature spermatozoa are released into the lumen of the seminiferous tubule.

The gross morphological features of spermatozoa vary quite considerably from species to species, although each possesses certain common features. One of these is the head region. This part of the spermatozoon contains the condensed nucleus with its associated acrosomal cap and post-acrosomal region. The latter region of the sperm is united to the tail which is itself subdivided into the middle piece and this is joined to the head by a short neck segment. More caudally the principal and end pieces of the tail are located.

TESTICULAR MORPHOLOGY AND FUNCTION DURING THE POSTNATAL PERIOD IN RODENTS

The situation observed in rodents with regard to the sequence of events associated with spermatogenesis and the production of the ejaculate has been described in some detail in the rat, although less is known about the sequence of events in the mouse. In the latter species, spermatogenesis is first evident shortly after birth and is similar to that observed in other laboratory rodents. The transformation of the prespermatogonia into type A spermatogonia occurs shortly after the initiation of spermatogenesis and is observed within a few days of birth. By about 8–10 days postpartum, the first primary spermatocytes are observed, and the prespermatogonia are no longer seen. The spermatocytes undergo successive stages of meiosis and differentiate into spermatids. Lumen formation within the seminiferous tubules is first evident only on or about postnatal day 14. A number of published detailed light and electron microscopic studies describe in considerable detail each stage of the spermatogenic cycle in the mouse and indicate that there are at least 12 clearly defined stages,[43] briefly summarized by Sinowatz et al.[44]

As in the human testis (indicated above), one of the most obvious morphological features of the adult mouse testis is the presence of a fibrous capsule (the *tunica albuginea*) from which connective tissue septae extend inward toward the medullary hilar region of the testis. These septae progress to form the *mediastinum testis* through which the blood vessels enter the testis, and the efferent ducts leave it as they pass toward the epididymis. Also clearly evident at this time is compartmentalization of the testis, with the formation of the seminiferous tubules and the region between them, termed the interstitial region. As noted previously, the Sertoli cells are exclusively located within the seminiferous tubules (see below), while the Leydig cells are specifically located within the interstitial compartment (see below). In addition to the Leydig cells, the interstitial compartment also contains blood and lymphatic vessels, nerve fibers, and macrophages. It has also been noted that the endothelial lining of the capillaries within the testis do not possess fenestrations, while the lymphatic vessels are only incompletely lined by endothelium.

The seminiferous tubular compartment is bounded by a tubular basement membrane that contains *myoid cells*, the contractions of which provide the forces that propel the enclosed mature spermatozoa and tubular fluid toward the excretory ducts of the testis. No nerve endings are present in or near this layer, and it appears that their contractility is generated intrinsically and that they provide a critical component of the blood–testis barrier in these species. Once within the latter, the secretions of the various accessory sex glands are added to the tubular fluid to form the definitive ejaculate.

The accessory sex glands in the mouse are similar in most regards to those present in other mammals and consist of the prostatic complex and the bulbo-urethral glands.[45] Also present are the seminal vesicles and ampullary glands that form from the caudal end of the mesonephric duct. The prostate is subdivided into a number of individual lobes (i.e., ventral prostate, dorsolateral prostate, and coagulating gland that forms the anterior prostate), which partially surround the prostatic region of the urethra.[44,46,47]

THE COAGULATING GLAND IN THE MOUSE

As its name suggests, the paired coagulating glands secrete certain substances into the ejaculate that cause it to coagulate when it enters and interacts with the components of the female reproductive tract. Curiously, this particular feature is not observed in the rat. These spindle-shaped glands lie in proximity to the concave smooth surface of the seminal vesicles and are fixed to the latter by connective tissue. The tubules of these glands are lined with simple columnar epithelium, while the apical part of the constituent cells contains numerous small secretory granules. Each gland has two ducts that enter the dorsal wall of the neck of the bladder.[44,48–50]

THE SERTOLI CELLS

It is now believed that the Sertoli cells of the mouse and human are probably one of the first cells to be induced from the *indifferent* state by the *Sry* gene (termed *Sry* in the mouse and *SRY* in the human), and they seem to play a critical role in initiating testicular formation.[4] As noted previously, the Sertoli cells synthesize AMH.

This hormone-like substance is responsible for stimulating the regression of the paramesonephric or Müllerian ducts that would otherwise give rise to the various components of the female reproductive duct system[51] (i.e., the oviduct, body, and cervical region of the uterus and possibly the upper one third or quarter of the vagina).[52] The Leydig cells appear about a day later. In addition, the environment within which PGCs are located at this stage determines their eventual fate. If the germ cells are enclosed within testicular cords, they then enter mitotic arrest. This is a characteristic feature of spermatogenic cells. Otherwise they enter meiosis. These cells subsequently arrest in meiosis, as invariably occurs in germ cells that are progressing through oogenesis.[53]

When germ cells fail to enter the genital ridge during their migration and are located, for example, in the adrenal (in an XY embryo), the germ cells behave like oogonia and will enter meiosis. This does not occur when they are located within testicular cords.[4] Some cases of XY sex reversal in humans and mice have been attributed to mutations in the *SRY* or *Sry* gene, respectively, thus confirming that it is necessary for testis determination. It has also been established that this gene is located on the Y chromosome and is believed to be the only gene to possess this critical role. Other evidence suggests that the *Sry* gene is expressed only between about E10.5 and E12 days *post coitum* (dpc) of mouse development, exclusively in the cells of the genital ridge.[54] In the light of additional in vitro experiments, it is now considered that other factors are probably necessary to complete Sertoli cell differentiation.[55] These studies[54,55] also indicate that the *Sry* gene is not required for the long-term maintenance of testis-specific gene expression.[56] Information from these studies indicates that by about E11.5 dpc *Sry* expression in the testis has significantly declined. Published work additionally indicates that cell–cell interactions between immigrating mesonephric cells (e.g., those believed to develop into the peritubular myoid cells) and presumptive Sertoli cells

are essential for the subsequent development of the testicular cords and for the further differentiation of the Sertoli cells.

Information from experimental studies in mice indicates that testicular cord formation and ovarian differentiation can occur in the almost complete absence of germ cells, for example, in certain mouse mutations,[57,58] after irradiation of pregnant females,[59] and after the treatment of undifferentiated gonads and ovaries with busulphan.[60] Testicular differentiation also appears to occur in the absence of the influence of gonadotrophins[61] and steroid hormones.[62] It is also relevant to note here that embryos with extreme mutations at the *W* locus, in which the genital ridges are also devoid of PGCs, are nevertheless still capable of developing testes normally.[63]

In the human and mouse embryos, the exact mechanisms leading to testicular cord formation have yet to be established. It appears, however, that the formation of an intact basal lamina that surrounds the testicular cords is an essential requirement in both of these species, and the same appears to be the case in all the other species in which the males have so far been studied.[64–67]

Throughout the period of testicular cord differentiation, the proximal ends of the cords maintain their connection to the mesonephric-derived rete tissue (sometimes termed the *rete blastema*). It therefore appears likely that the mesonephric cells maintain contact with both the intratubular as well as the interstitial space and therefore probably play a role in the activities of both the Sertoli and Leydig cells. It also appears that increased vascularization is associated with the growth of the testicular anlage. It has been suggested from detailed studies in the pig that the ingrowth of these capillaries may stimulate the differentiation of the testicular cords, as the first area where differentiation is observed in this species is also the first part of the testis to become vascularized.[65,66] With regard to the function of the testicular cords, experimental evidence suggests that these require the almost complete separation of the testicular from the mesonephric tissue.[68]

A negative feedback loop exists between the hormone *inhibin* (produced by the Sertoli cells in the testis and granulosa cells of the ovarian follicle) and follicle-stimulating hormone (FSH) released by the pituitary. Thus, an elevated level of inhibin from either source results in the suppression of FSH release from the pituitary. This represents a similar negative feedback loop to that between the secretion of testosterone by the Leydig cells of the testis and the release of luteinizing hormone (LH) from the anterior pituitary (see below).

THE LEYDIG CELLS

These are the androgenic steroid-producing cells of the testis and are located within its interstitial compartment. The Leydig cells are readily recognized between the testicular cords because of their rounded appearance and dense cytoplasm that stains by the periodic acid-Schiff (PAS) technique,[69] although their exact source within this compartment has yet to be definitively established both in the mouse[42] and the human.[6,70] The principal period during which they proliferate is between puberty and sexual maturity,[42] and the rise in the level of testosterone production appears to be correlated with an increase in their volume and number.

Though in the human this compartment is largely occupied by extravascular space containing a protein-rich fluid, in other species this space is almost completely filled by the Leydig cells.[8] It is exclusively within these cells that the endocrine function of the testis resides. They principally synthesize testosterone, and this is required locally for the maintenance of spermatogenesis in the seminiferous tubules. Testosterone is also essential for the normal functioning of the male accessory sex glands and for the maintenance of the male secondary sexual characteristics.[6,71]

The steroid functioning of the Leydig cells appears to be principally dependent on the secretion of LH by the anterior pituitary, while the exact role of *prolactin* in testosterone production has yet to be established. The testosterone that is produced does not appear to be stored in the form of secretory granules but is synthesized and released as necessary. Evidence also suggests that there may be a local feedback mechanism involving a peptide produced by the Sertoli cells and that this has a regulatory effect on the activity of the Leydig cells with regard to testosterone production. The LH

released by the anterior pituitary is regulated via a negative feedback loop so that increased levels of testosterone synthesized by the Leydig cells suppress the release of pituitary LH. By the same mechanism, low levels of circulating testosterone result in an increase in the release of LH from the anterior pituitary.

By contrast, in the condition of *testicular feminization*, due to the lack of androgen receptors in XY individuals, the number of Leydig cells is dramatically increased, and the concentration of circulating gonadotropins is higher than normal. This is one of a range of androgen-insensitivity syndromes[28,72] and would appear to draw attention to the importance of gonadotropins in Leydig cell differentiation and maintenance.[73]

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Life Science

Histologic Basis of Mouse Endocrine System Development

A Comparative Analysis

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