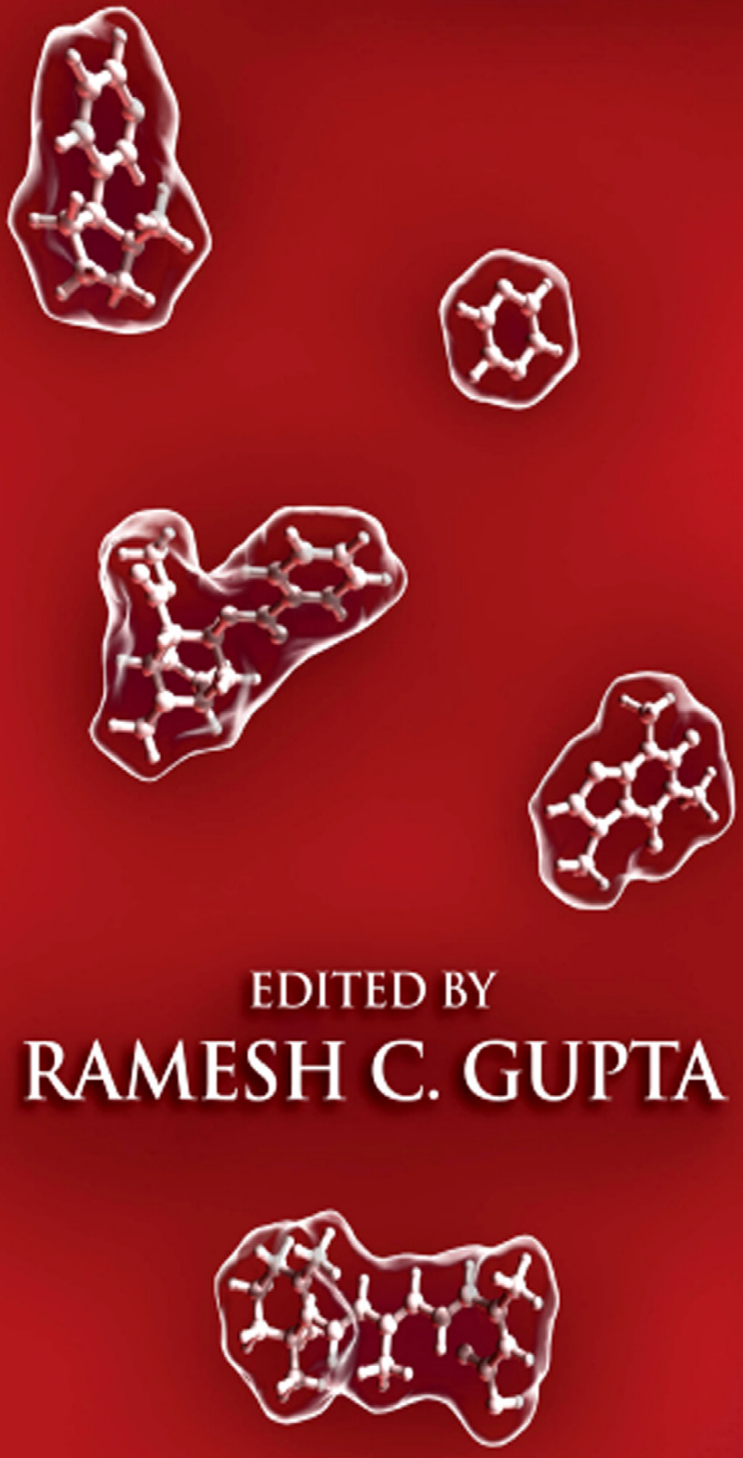
REPRODUCTIVE AND DEVELOPMENTAL TOXICOLOGY



EDITED BY

RAMESH C. GUPTA

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This book is dedicated to my wife Denise, daughter Rekha, and parents the late Chandra and Triveni Gupta.

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Foreword

This book, *Reproductive and Developmental Toxicology*, presents one of the most comprehensive and thorough treatments of the complex discipline of toxicological phenomena in reproducing and developing organisms available. The focus is obviously often on human species, which is quite understandable, but the book also covers other species, from organisms used for toxicology testing to related aspects of wildlife species. The book surveys a large number of different chemicals, from pharmaceuticals to environmental pollutants, and various experimental systems at all levels of biological organization. We anticipate that this book will be heavily used as a handbook for critically evaluated information that may be not so easily available from other sources.

There are several reasons why such a wide and thorough collection of authoritative reviews and surveys is useful, even imperative. The first reason is the very extraordinary nature of the subject: the developing organism and its creation. Adults of reproducing age "get the ball rolling", so to speak, but by no means is the new organism a small adult. It could even be said that there is no such thing as a developing organism, but an organism that is constantly and often rapidly changing, with various and variable characteristics at each point in time. It is a moving target for research and the dimension of time has always to be taken into consideration.

Development is manifest at all levels of inquiry: expression of genetic programs at specified stages, consequent changes in the patterns of nucleic acid messages, proteins, enzyme activities, signal transduction systems and so on, as well as formation and modification of anatomical structures and physiological functions. And ultimately, this finely tuned marvel of creation of a new individual could be disrupted at any stage of development, in various ways and by various forces, by physical, chemical and biological insults. The grand goal of the research on reproductive and developmental toxicology is to understand the interplay between exogenous, potentially harmful factors and endogenous, intrinsic molecular, physiological and anatomical determinants, which may ultimately result in derangements in reproduction and development. The epitome of such a deranged development was the thalidomide catastrophe about 50 years ago, which had and still has far-reaching consequences in basic research, drug development and regulatory pharmacology and toxicology.

As toxicologists and pharmacologists, we used to think that chemicals most often cause their effects via specific target molecules, receptors, enzymes, regulatory factors and so on. However, the appearance of such targets in the developing organism depends on developmental programs, which dictate appearance and disappearance of specific molecular effectors and modifiers. Consequently, if a specific target is still "sleeping" at a certain stage in development, a chemical affecting that specific target does not cause an effect. Toxicity mechanisms elucidated in adults do not necessarily apply in developing organisms.

A developing organism does not exist on its own; it is dependent on its mother, and there are unique structures such as yolk sac and placenta taking care of certain functions during pregnancy. The placenta both connects and separates mother and fetus, and after birth its function has been fulfilled. From a toxicological point of view, the placenta has a central function: it controls the movement and access of chemicals from mother to fetus. Although we know now that the placenta is not a barrier in the old meaning of the word, we still use this misnomer. It is imperative to understand the role of the placenta in the kinetics and dynamics of chemicals, because only then we can fully assess potential hazards and risks to a developing organism.

Up to this day many, perhaps most, reproductive and developmental toxicants have been detected after human exposures. However, the best way to avoid such tragedies should be prevention: to detect potential developmental toxicity in animals before human exposures. Since the thalidomide tragedy, drugs and many other chemicals with intended or unintended human exposures have had to be screened in animal experiments. Recently also a few *in vitro* testing systems have been validated for the same purposes. Animal experiments have their own drawbacks, including sometimes very large and partially unknown or unexplained

interspecies differences, and increasingly influential ethical issues. The main problem of *in vitro* testing systems is that they can never represent the whole complex organism, only some rather limited processes, and thus they need extensive validation to be reliable indicators for developmental hazards and risks. A significant way to avoid difficulties inherent in animal or *in vitro* studies is the thorough characterization of physiological and pathological development and the identification of rate-limiting processes and mechanisms via which toxicants may affect normal development.

The most important humane reason to emphasize the significance of continuous research in reproductive and developmental toxicity is the simple fact that damage in early life, if permanent, will be with the affected individual for the rest of their life. This is also the principal reason why research efforts have to be directed towards preventive, anticipatory tools and actions. The ultimate goal is to prevent the exposure of reproducing adults and developing individuals to potentially harmful toxicants by reliable and predictive toxicity testing, which employs the most modern *in silico, in vitro, ex vivo* (and *in vivo*, if possible and necessary) tools in an integrated framework of hazard identification and risk assessment.

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Section 1

General

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CHAPTER

1

Introduction

Ramesh C. Gupta

INTRODUCTION

Unsuccessful conception and adverse pregnancy outcomes have likely occurred since the inception of life. The etiology of such disappointing events can often be attributed to common factors such as malnutrition, hyperthermia, or a stressful environment at home or at the workplace. In addition, exposure to biotoxins, chemical toxicants, radiation or multiple factors seems to be involved in infertility, miscarriage and birth defects. A single factor or a combination of these factors can exert deleterious effects on male and/or female reproductive performance and on the mother, placenta or conceptus after conception. Homeostatic maintenance of human and animal/wildlife species requires proper function of the male and female reproductive systems, and development of offspring.

Reproductive and developmental toxicology is a very complex subject because of continuous changes taking place in the mother, placenta and the unborn. Exposure of the developing organism to chemicals can occur *in utero* or through the mother's milk or contaminated food. In general, it is believed that developing organisms are more sensitive than adults to the toxic effects of chemicals because of limited defense and detoxifying mechanisms. In particular, the nervous and reproductive systems may be more vulnerable to the toxic insult of chemicals due to incomplete blood–brain and blood–testes barriers. Compelling evidence suggests that *in utero* or early postnatal exposure to chemicals not only damages the developing organism, but can predispose an individual for the development of devastating diseases like diabetes, metabolic syndrome, Alzheimer's or Parkinson's in later life.

Toxicological problems related to reproductive and developmental systems have been recognized for centuries, but this area of toxicology has received enormous attention since the thalidomide incident. During the period of 1957–1961, thousands of pregnant women around the world received thalidomide for morning sickness. More than 10,000 children, exposed *in utero* to thalidomide during the first trimester of gestation, were born with a variety of severe birth defects, mainly phocomelia and amelia. Other anomalies related to thalidomide syndrome involved eyes, ears and the central nervous system. From this tragedy, with exhaustive efforts over half a century, scientists learned that: (1) wide species differences exist due to unknown factors, (2) the period of exposure is crucial for expression of teratogenicity, and (3) thalidomide exerts multifaceted effects through multiple mechanisms, although, we are still far from understanding the exact mechanism of teratogenicity. Presently, thalidomide and its analogs are available on the market for indications in leprosy, Crohn's disease, HIV, multiple myeloma and vascular disorder, but of course not prescribed for women who are pregnant or trying to get pregnant.

In another incident, methylmercury was involved in Minamata disease in Japan affecting approximately 3,000 people after consumption of contaminated fish during the late 1950s to the mid-1960s. In the early 1970s, more than 10,000 people died and 100,000 suffered permanent brain damage in Iraq by consuming "wonder wheat" imported from Mexico that was treated with methylmercury as a fungicide. In both incidents, offspring of mothers exposed to methylmercury suffered from severe malformations, cognitive impairment, and behavioral disorders, including "quiet baby syndrome". Because of the catastrophic effects of Minamata disease, the Japanese government has established the "National Institute for Minamata Disease" for biomonitoring and surveillance of mercury exposure to avoid future cases.

Following the thalidomide tragedy, drug safety efforts were intensified throughout the world; however, although presently more than 80,000 chemicals are on the market, used alone or in combinations, only 200 of them have been tested for toxicity and safety. Developmental and reproductive toxicity testing (DART) in animals has been a vital component of the drug development process for humans since the late 1940s. Currently, this set of non-clinical studies in animals is required for drug approval by regulatory agencies, such as the US Food and Drug Administration (FDA), the Organization for Economic and Cooperative Development (OECD), the Japan Pharmaceutical Manufacturers Association (JPMA), and other such agencies in many countries. Currently, many associations (the Pharmaceutical Manufacturers Association, the European Federation of Pharmaceutical Industries Association, and the Japan Pharmaceutical Manufacturers Association), professional organizations (the Society of Toxicology and its specialty section on Reproductive and Developmental Toxicology, the Teratology Society and the International Federation of Teratology Societies) and regulatory agencies (primarily

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from the USA, Europe, and Japan) are actively engaged in drug safety to avoid reproductive and developmental effects. In this context, the International Federation of Pharmaceutical Manufacturers Association (IFPMA) plays a pivotal role in bringing together the regulatory authorities of the USA, Europe, Japan and elsewhere. In the USA, agencies including the Consumer Product Safety Commission, the US Environmental Protection Agency, the US Food and Drug Administration, the US Department of Agriculture, the Agency for Toxic Substance and Disease Registry, the National Toxicology Program, the National Institute of Environmental Health Sciences, the National Institute for Occupational Safety and Health and the Occupational Safety and Health Administration, and in Europe the OECD and REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), play pivotal roles in safety evaluation of non-pharmaceutical chemicals.

It is worth mentioning that developmental and reproductive toxicity risk assessment criteria differ from country to country, and the International Conference on Harmonization (ICH) and related agencies take an active part in dealing with such disparities. The objective of all these regulatory agencies is to identify reproductive and developmental hazards and to ensure the safety of drugs and chemicals.

This book, *Reproductive and Developmental Toxicology*, provides extensive coverage of safety evaluation of new pharmaceutical compounds and risk characterization of chemicals using the guidelines of the agencies listed above.

The complexity of reproductive and developmental toxicity involves many variables, including species, gender, developmental stage, diet, genetic polymorphisms, environmental and many other factors. Pregnant women, the unborn, infants and toddlers constitute unique populations with greater vulnerability in terms of sensitivity to chemicals. Even functional foods including black tea, coffee, etc. can cause developmental effects if consumed in excess during gestation.

It is well established that environmental and genetic factors in relation to chemical toxicity have changed significantly in the last 50 years. This is partly due to the flood of chemicals (therapeutic drugs, industrial chemicals and environmental pollutants), greenhouse gases and global warming. Alcohol, smoke, illicit drugs and anticonvulsants are among the most frequently encountered reproductive and developmental toxicants. These substances, along with many others, cross the placental barrier easily and can lead to a variety of effects, including intrauterine growth restriction (IUGR), preterm birth and spontaneous abortion.

Environmental contaminants, such as PCBs and brominated flame retardants, and recently bisphenol A, phthalates, perfluorooctanoic acid, pesticides, lead in toys (toxic toys), cadmium and zinc in imported jewelry, and high levels of cadmium in drinking glasses and dishes, have raised serious concerns about adverse health effects in general and reproductive and developmental effects in particular. The current concern about "Toxic Childhood" in "Toxic America" is real and the community as a whole has no choice but to face the challenges of the 21st century to minimize chemical exposure.

Each year approximately 3% of babies in the USA are born with birth defects that are life-threatening. One of the most common human birth defects is neural tube defects (NTDs), due to failure of neural tube closure, often resulting in anencephaly, exencephaly and spina bifida. Although, the etiology of NTDs is complex, chemical agents (antiepileptic drugs, thalidomide, folate antagonists, etc.), in addition to genetic and environmental factors, appear to be involved.

Today's advanced technologies allow biomonitoring of chemical (therapeutic and environmental concern) residues at parts per billion or parts per trillion in biological tissues and fluids. In recent investigations, 10,000 babies were examined and more than 200 chemicals were found in the umbilical cord. On the one hand, the presence of a chemical in the cord blood does not prove the chemical is harmful to the unborn; on the other hand, its harmful effects cannot be ruled out unless proven safe based on toxicity testing. In essence, every chemical is safe unless proven toxic. Molecular toxicology offers novel biomarkers and sensitive endpoints of cellular and molecular damage (biochemical, neurochemical or histopathological) to the fetus that are particularly useful in reproductive and developmental toxicity and safety testing. *In vitro*, *in vivo* and *in silico* models, national and international guidelines for toxicity testing, and international harmonization in risk assessment criteria are necessary for the safety evaluation of chemicals and drugs. Pharmacokinetics/toxicokinetics and physiologically based pharmacokinetics of drugs/toxicants seem to differ substantially in male vs. female, and more so in pregnant vs. non-pregnant; and therefore special attention should be paid when dealing with pregnancies, and fetal, neonatal and pediatric populations. Current technologies such as ultrasound, MRI and micro-CT imaging aid in an early diagnosis of any malformations in embryonic-fetal development.

Reproductive and Developmental Toxicology is the single most comprehensive resource on this subject, comprised of more than 80 chapters, which are arranged into 15 sections. The book is prepared with a user-friendly format for academia, pharmaceutical industries and regulatory/governmental agencies. Standalone chapters are provided on major topics, so the reader can easily find the required information. The volume covers many novel topics related to reproductive and developmental toxicants, especially topics of current concern, such as endocrine disruptors, pesticides, industrial solvents, metals, bisphenol A, phthalates, nanoparticles, nutraceuticals, pharmaceuticals, phytoestrogens, mycotoxins and zootoxins. Ten chapters are offered in Section XI on special topics, including stem cells, toxicogenomics, metabolomics, epigenetic regulation, cell signaling mechanisms, neuroinflammation, and mitochondrial dysfunction in reproductive and developmental toxicity. Multiple chapters offer state-of-the-art techniques, including ultrasound, magnetic resonance and micro-CT imaging for prenatal diagnosis of developmental anomalies. Atlas-style coverage of toxicologic pathology is presented for testing and screening of chemicals having the potential for reproductive and developmental toxicity. Since the placenta is the key to the success of pregnancy, extensive coverage of placental toxicity is provided with five chapters, dealing with placentation in humans and rodent species, placental role in fetal programming and biocommunication between mother and fetus, placental structure, function and barrier, significance of transporters and other molecular mechanisms in the feto-placental unit, and toxicologic pathology of a variety of drugs, chemicals and biotoxins. Finally, the last section of the book offers multiple chapters describing reproductive and developmental toxicity and endocrine disruption in domestic, wildlife and aquatic species.

The contributors of this book are highly qualified and considered authorities in toxicology in general and reproductive and developmental toxicology in particular. Their hard work and dedication to this book is greatly appreciated. The editor expresses his gratitude to Robin B. Doss and Kristie M. Rohde for technical assistance, Alexandre Katos for the cover design and Denise M. Gupta for indexing. Last but not least, the editor immensely appreciates the tireless efforts of publishing editors April Graham, Nancy Maragioglio and Kirsten Chrisman at Academic Press/Elsevier for their various roles in the preparation of this book.

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CHAPTER

2

Reproductive anatomy and physiology

Timothy J. Evans and Vekataseshu K. Ganjam

INTRODUCTION

In order for one to fully appreciate how xenobiotics can adversely affect reproductive function, including development, it is necessary to have some understanding of the coordinated sequence of events and physiological processes involved. Normal reproduction will be reviewed in this chapter to provide anatomical and physiological bases for the discussions of specific mechanisms of action and reproductive toxicants in the other chapters of this book. Although the emphasis of this chapter will be on human reproduction, many of the same principles are applicable to reproductive processes in other mammals, as well as other classes of vertebrates.

Unfortunately, space constraints limit the amount of information which can be presented in this chapter, and many of the presented topics cannot be discussed at great length. If additional information is required for better understanding of the subject matter, there are several excellent textbooks which provide an overview, including detailed illustrations, of the basic reproductive anatomy and physiology of humans (Berne et al., 2004; Netter, 1997; Piñón, 2002), as well as animals (Senger, 2007). There are also a number of book chapters in other toxicology texts which cover this information, as it applies directly to exposures to toxicants (Evans, 2007; Foster and Gray, 2008). Other references can be consulted for more in-depth discussion of specific cells or organs involved in the reproductive process (De Jonge and Barratt, 2006; Payne and Hardy, 2007; Skinner and Griswold, 2005). The reader is also directed to references cited in this chapter (many of which are available online) in order to gain additional insight into the specific topics being discussed.

IMPORTANT DEFINITIONS AND CONCEPTS

Reproduction

Reproduction in humans, as well as domestic, wild and laboratory vertebrates, encompasses the wide range of physiological processes and the associated behaviors and anatomical structures necessary for the birth of the next generation of a given species (Evans, 2007; Senger, 2007). Those physiological processes involved specifically in human reproduction are illustrated in Figure 2.1 and generally include the following (Evans, 2007):

1. Gametogenesis (production of sperm or ova) and the pre- and peripubertal changes leading up to its onset.
2. Release of gametes (i.e., sperm transport/maturation, libido/courtship, penile erection, intromission/copulation, emission and ejaculation of semen, and ovulation of an oocyte).
3. Formation of the zygote (i.e., sperm storage, capacitation, and processes leading to fertilization or union of a single sperm with an egg).
4. Embryonic and fetal development during pregnancy or gestation (i.e., activities related to the initiation and progression of zygote cleavage, blastocyst formation, separation of the germ layers, placentation, neurulation and organogenesis.
5. Parturition or "birth" of a single or multiple offspring.
6. Lactogenesis and lactation for the postpartum nutrition of offspring.

All of these processes are potential targets for reproductive toxicants present in the environment, workplace or home.

Hormones and hormone receptors

The term "hormone" classically refers to a substance which is secreted into the circulation by a ductless gland and which alters the function of its target cells (Hodgson *et al.*, 2000). While the traditional "endocrine" aspect of hormone action involves organ-to-organ signaling (and in the case of mammalian pregnancy animal-to-animal signaling), it is recognized that hormones can also be involved in "paracrine" (cell-to-cell) communication and signaling pathways within the same cell in which they were produced ("autocrine" function) (Evans, 2007). In vertebrates there are a wide variety of different hormones involved in reproductive function. The major reproductive hormones are generally grouped according to their basic molecular structure and include amino acid derivatives (e.g., dopamine or prolactin inhibitory factor and melatonin); peptides (e.g., oxytocin, adrenocorticotropin hormone or ACTH, corticotropin releasing factor or hormone or CRF/CRH, gonadotropin releasing hormone or GnRH, and

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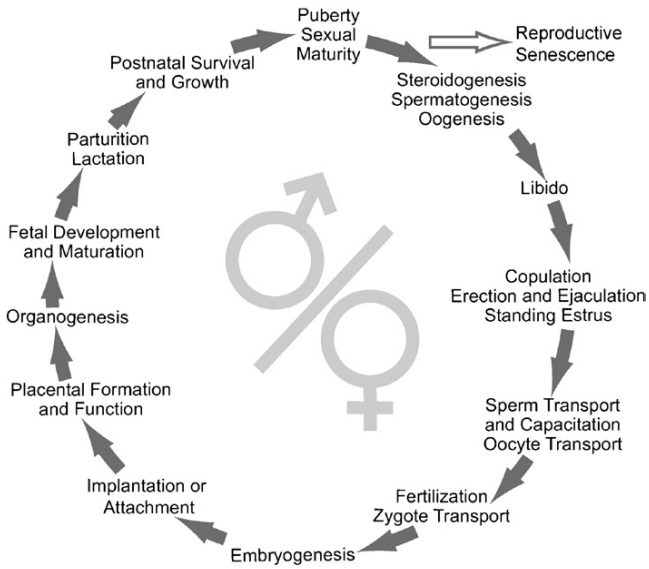

FIGURE 2.1 The continuum of developmental stages and reproductive functions taking place in males and/or females, as well as the embryo and fetus, are shown schematically and illustrate the complexity of reproduction in mammalian species, especially in humans, where additional behavioral, psychological, social and environmental factors, as well as eventual senescence, can come into play. This figure was adapted, with permission, from Evans (2007). Modifications and artwork were courtesy of Don Connor and Howard Wilson.

thyrotropin releasing hormone or TRH); proteins (e.g., activin, inhibin, insulin-like growth factors, prolactin and relaxin); glycoproteins (e.g., follicle-stimulating hormone or FSH, luteinizing hormone or LH, and thyroid-stimulating hormone or TSH or thyrotropin); steroids (e.g., androgens, estrogens and progestagens); and eicosanoids, which include prostaglandins.

The actions of hormones on their targets are generally mediated through receptors which initiate or inhibit some sort of signal transduction pathway or are required for hormone-induced alterations in gene expression. Hormone–receptor interactions can be modulated by a number of factors, including the amount of hormone present, the affinity of the hormone for the receptor, receptor density and occupancy and interactions with other hormones, receptors and hormone-receptor complexes, as well as a variety of endogenous co-activators and inhibitors (Bigsby *et al.*, 2005; Evans, 2007; Genuth, 2004a). It should be evident from the topics covered in this textbook that various xenobiotics are also capable, under certain exposure conditions, of modulating the interactions between endogenous hormones and their receptors.

Gonadal steroid hormones and their "nuclear" receptors

As has been reviewed by the authors previously (Evans *et al.*, 1997), the basic structure of steroid hormones consists of four rings labeled as A, B, C and D. The various members of this hormone class differ from one another with respect to the location of double bonds and types of functional groups attached to the ring structure. The major gonadal steroids are also referred to as the "sex" steroids and include androgens (i.e., androstenedione, testosterone and dihydrotestosterone, which is the 5α-reductase conversion product of testosterone in the testes and selected non-gonadal tissues), estrogens (i.e., estradiol and estrone) and, for the purposes of this chapter, progesterone and other endogenous progestagens. Mineralocorticoids, glucocorticoids and progestagens are all 21-carbon compounds. Androgens are 19-carbon compounds, and estrogens contain 18 carbons. In the classical Δ^4 biosynthetic pathway for endogenous steroids, cholesterol is the steroid precursor, and the rate-limiting step in steroidogenesis is cholesterol transfer within the mitochondria, which is mediated by steroidogenic acute regulatory protein (Stocco, 2007). Cholesterol is cleaved and converted to the progestagen, prenenolone, which is converted to progesterone by 3β-hydroxysteroid dehydrogenase. Androstenedione is synthesized from progesterone by the actions of several enzymes, including 17-hydroxylase, and can be converted to testosterone by 17β-hydroxysteroid dehydrogenase. Androgens are converted to estrogens by aromatase, a member of the cytochrome P450 (CYP) family of enzymes. Androstenedione is converted to estrone, and testosterone is converted by aromatase to estradiol. It is also possible in the Δ^4 steroidogenesis pathway for estradiol to be synthesized from estrone via the actions of 17β-hydroxysteroid dehydrogenase (Evans *et al.*, 1997).

In appropriate cell types, mineralocorticoids and glucocorticoids can be synthesized from progesterone. Interestingly, both of these types of steroid hormones can also interact with the promiscuous mineralocorticoid receptor. Isoforms of 11β-hydroxysteroid dehydrogenase are present in many different cell types to regulate the relative proportions of the active and inactive forms of glucocorticoids (i.e., cortisol and cortisone, respectively, in humans). This regulation is important from the perspective of mineralocorticoid activity, as well as the modulation of the adverse effects of glucocorticoids on reproduction and other physiological processes (Hardy and Ganjam, 1997).

The gonadal steroids facilitate the development and regulation of reproductive function in humans and animal species, in large part by interacting with (i.e., functioning as ligands for) receptors which are members of the steroid/thyroid ("nuclear") receptor superfamily, the largest family of transcription factors in eukaryotic systems (Evans, 2007; Genuth, 2004a; Tsai and O'Malley, 1994). Receptors in this superfamily are large oligomeric proteins (Genuth, 2004a), which generally consist of six domains (A/B, C, D, E and F) (Tsai and O'Malley, 1994). Although specific portions of the gonadal steroid nuclear receptor molecules can interact with a variety of co-activators as well as inhibitors, the most important domains of these receptors are generally considered to be those involved in transactivation (N-terminal A/B domain; also C-terminus in estrogen receptors); DNA-binding and hormone–receptor complex dimerization (middle portion containing two helical zinc fingers; C domain); and hormone (ligand) binding (C-terminus; E domain) (Bigsby *et al.*, 2005; Genuth, 2004a). While androgen, estrogen and progesterone receptors, which are members of the steroid/thyroid superfamily, are often thought of as being exclusively nuclear in their location, these receptors can also be located in the cytoplasm of some cells. Cytoplasmic and nuclear gonadal steroid receptors can be bound to a variety of different heat shock proteins, which interact with the receptor's hormone-binding domain. Heat shock proteins can act as "blocking" molecules and are displaced by hormones binding to the receptors (Bigsby *et al.*, 2005; Genuth, 2004a) or as "chaperones" involved in receptor turnover and "trafficking" of these receptors between the nucleus and cytoplasm (Evans, 2007).

There is reportedly a single type of androgen receptor which is a member of the steroid/thyroid superfamily. In contrast, there are two types of nuclear estrogen receptors

(ERα and ERβ), which are the products of distinct genes on separate chromosomes (O'Donnell *et al.*, 2001). ERα and ERβ differ in their amino acid structure, tissue distribution, affinity for selective ER modulators (SERMs) and their role in female (Britt and Findlay, 2002) as well as, somewhat surprisingly, male fertility (Evans, 2007; Hess, 2003; O'Donnell *et al.*, 2001). The nuclear progesterone receptor also has two isoforms, progesterone receptor A and progesterone receptor B (PRA and PRB, respectively), which differ slightly in their amino acid sequences and their interactions with co-activators, but, unlike ERα and ERβ, PRA and PRB are the product of a single gene (Brayman *et al.*, 2006).

Genomic and non-genomic mechanisms of action of gonadal steroid hormones

Traditionally, the receptor-mediated reproductive effects of gonadal steroids were thought to occur almost exclusively through interactions between homodimers of the hormone–nuclear receptor complexes and specific regions of DNA upstream from the basal promoter of a given gene, referred to as hormone response elements (HREs) or, more specifically, androgen and estrogen response elements (ARE and ERE, respectively) (Genuth, 2004a; Tsai and O'Malley, 1994). It is now understood that these "genomic" effects of gonadal steroids and their nuclear receptors, which involve alterations in gene transcription, can, in some instances, involve heterodimers of different nuclear steroid–receptor complexes, indirect binding of hormone–receptor complexes to DNA via proteins within a preformed transcriptional complexes and even ligand (hormone)-independent "activation" of nuclear gonadal steroid receptor molecules (Bigsby *et al.*, 2005; O'Donnell *et al.*, 2001; Thomas and Khan, 2005). In addition, it is also apparent that gonadal steroids can affect cellular function by non-genomic mechanisms of action involving changes in intracellular concentrations of ions, cAMP and its second messengers, and the mitogen-activated protein (MAP) kinase pathway. These non-genomic mechanisms are independent of the somewhat "time-consuming" alterations in gene expression traditionally associated with gonadal steroids and occur rapidly within seconds or minutes (O'Donnell *et al.*, 2001; Thomas and Khan, 2005). While the rapid, non-genomic effects of gonadal steroids most likely involve receptors bound to the plasma membrane, the specific identity and classification of these receptors remain unclear and might involve a number of different receptor types (Evans, 2007; O'Donnell *et al.*, 2001; Razandi *et al.*, 1999; Thomas and Khan, 2005; Warner and Gustafsson, 2006).

REVIEW OF NORMAL HUMAN REPRODUCTION

Historical perspectives and complexity of reproductive function

It should be evident from a review of Figures 2.2A, 2.2B and 2.2C that for well over 200 years the basic anatomical components required for human reproduction have been fairly well recognized and their primary functions understood. However, it has only been more recently that we have gained a more accurate understanding of the specific cellular, hormonal and molecular aspects involved in this process. Figure 2.1 demonstrates how reproduction is a complex and dynamic process involving precise coordination and integration of the functions of multiple organs within the body. The production of viable and functional gametes and their transport and union to form a zygote which develops into a healthy and fertile individual require that many stringent physiological and metabolic needs be met. A thorough understanding of the mechanisms involved in reproduction is absolutely essential in order to recognize which steps in the reproductive process are most susceptible to the adverse effects of potential toxicants.

Relevance of a basic understanding of human reproductive anatomy and physiology

It is necessary, from a clinical perspective, to identify what constitutes "normal" reproduction in order to recognize abnormal reproductive behaviors, function and morphologic changes in humans, as well as in wild, domestic and laboratory animals. It is also critical that one be able to understand the pathophysiological basis for reproductive abnormalities. Impaired reproductive function in humans associated with exposure to toxic amounts of xenobiotics necessitates the use of diagnostic, therapeutic and prognostic procedures, which require a thorough knowledge of normal reproductive anatomy and physiology (Evans, 2007). In addition, if we are to develop animal models for human reproductive diseases or are to extrapolate results of toxicology experiments performed with laboratory animals to human exposures to the same xenobiotics, we need to understand how human anatomy and/or reproductive physiology differs from that of the animals being used for modeling.

Neuroendocrine control of reproduction

In humans and animals alike, visual, olfactory, auditory and other sensory data are integrated within the brain and are reflected in endocrine events. The neuroendocrine functions of the pineal gland, hypothalamus and pituitary gland play an important role in the integration of the body's physiological processes, including reproduction, and are potential targets for toxicants (i.e., dioxins). The proper function of the hypothalamic–pituitary–gonadal axis facilitates development of the reproductive tract and endocrine regulation of spermatogenesis in the male and the menstrual or estrous cycle in the female. The onset of puberty and sexual behavior in males and females, the ability to achieve erection and ejaculation in males, and the normal progression of gestation, parturition and lactation in females are also facilitated by the secretions of the hypothalamus and pituitary gland (Evans, 2007; Evans *et al.*, 1997; Senger, 2007).

The hormones involved in the neuroendocrine control of reproduction are produced in several regions of the brain. Melatonin is produced in the pineal gland. The major hormones of reproductive interest which are of hypothalamic origin are dopamine, CRF, GnRH and TRH. Oxytocin is released from the posterior pituitary (neurohypophysis), and ACTH, FSH, LH, prolactin and TSH are synthesized and released from the anterior pituitary (adenohypophysis) (Evans, 2007; Evans *et al.*, 1997). The production and release of these hormones are regulated by various positive and negative feedback loops (Figure 2.3), which are potentially susceptible to the effects of hormonally active xenobiotics.

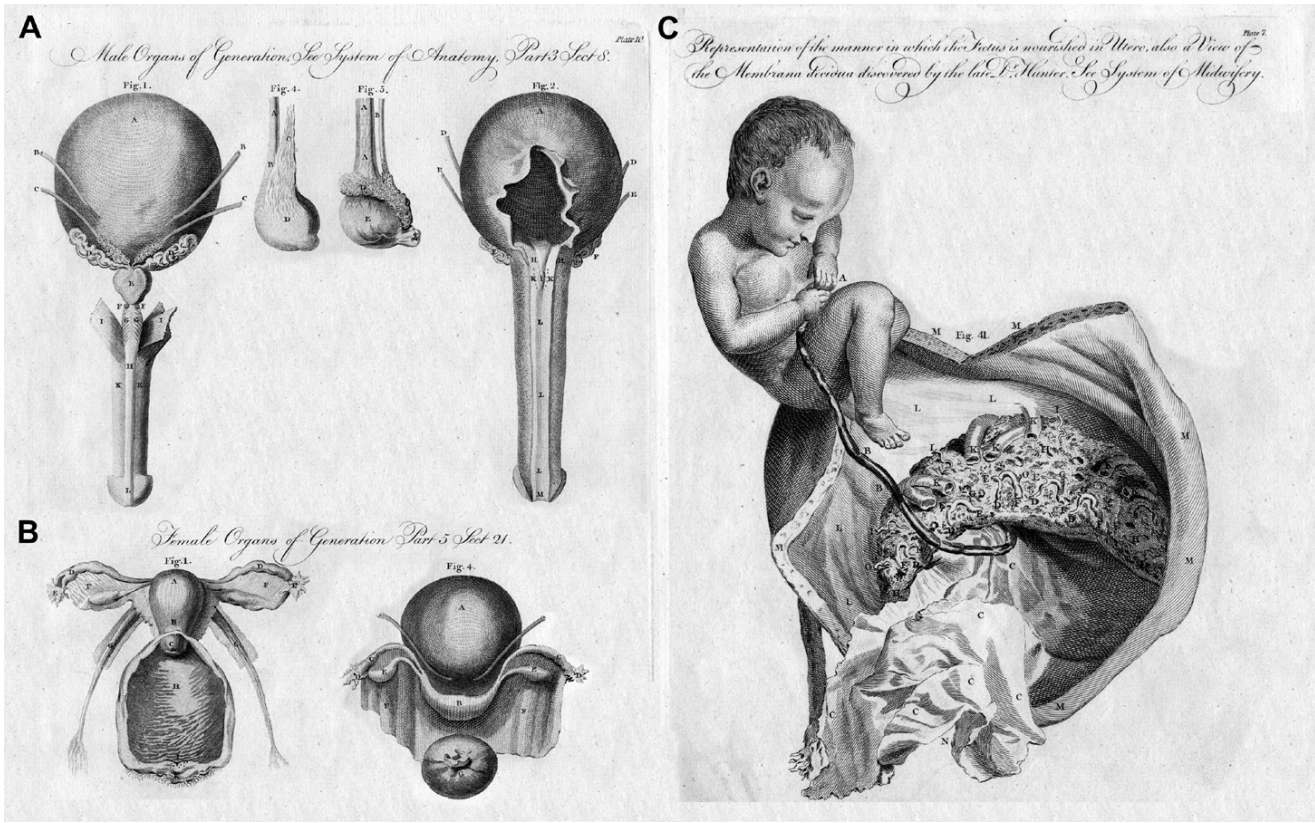

FIGURE 2.2 An artist's renderings, which were obviously not drawn to scale and which were published in the *Modern Universal Dictionary of Arts and Sciences* (also referred to as *Hall's Encyclopedia*) in or around 1798, show the male and female "organs of generation" and a representation of the "manner in which fetus is nourished *in utero*" in **A**, **B** and **C**, respectively. While, unfortunately, the original legends for these drawings were not available for review of the terminology, it should be clear, despite some departures from our current understanding, that there was a basic comprehension and appreciation of reproductive anatomy at the time and that people were keenly interested in learning more about these physiological processes. This figure will be explained in quite some detail, as it provides a historical basis for the extensive, subsequent investigation of the cellular, as well as subcellular and molecular processes involved in mammalian and, more specifically, human reproductive function. In **A**, the key anatomical components being demonstrated in Fig. 1 are posterior views of the urinary bladder (A), showing the entry of the ureters (B) into the bladder; the ductuli or ducti deferens (C) and their expanded distal extremities (i.e., the ampullae), which are considered accessory sex glands in men; and the other male accessory sex glands, including the seminal vesicles (D), the prostate gland (E) and bulbourethral or Cowper's glands (F). The position of the seemingly erect penis (most likely straightened for display purposes), with the foreskin and, possibly, the fascial and portions of the muscular layers removed, is not one which would be observed *in situ* (image of *in situ* anatomical arrangement not shown). If the pelvis were present, the pelvic urethra would form an approximately 90° angle with the penile or cavernous urethra and would be directed away from the reader. The "penile" structures shown from an inferior view include the bulbus urethrae covered by the bulbocavernosus muscles (G); the corpus spongiosum, which surrounds the penile urethra (H); the paired ischiocavernosus muscles (I); what appear to be the penile corpora cavernosa (K); and the distal end of the urethra surrounded by the glans. In Fig. 2 of **A**, the urinary bladder (A) and the ureters, ductuli deferens and seminiferous vesicles (D, E and F, respectively) are observed from an anterior view, and the "penile" structures in this image, which would be directed towards the reader in the presence of a pelvis, can be evaluated from a superior view. Important structures on the floor of the penile urethra (L), which terminates at the external urethral orifice located within the glans (M), include the seminal colliculus and the orifices of the ejaculatory ducts (I), as well as the multiple orifices of the prostate gland (K). The testis (D) in Fig. 4 appears to be covered by an intact parietal tunica vaginalis (i.e., a protective connective tissue structure which has internal or visceral and external or parietal components), with the cremaster muscle (C) and components of the spermatic cord (A and B) shown. The parietal tunica vaginalis appears to have been removed from the testis (E) in Fig. 5, which is viewed from the lateral perspective, showing portions of the epididymis (C and D), as well as the vascular components of the spermatic cord (A) and the ductus deferens (B). In **B**, the key anatomical components of the female reproductive tract and nearby organs are shown from frontal (Fig. 1) and posterior perspectives (Fig. 4). The fundus (A), body (B) and cervix or internal cervical os (C) of the simplex human uterus are illustrated in Fig. 1 and connect with the uterine or Fallopian tube or oviduct (D) and its terminal infundibulum, with the associated fimbriae and ostium (E) above, and with the vagina (H) below. The urethral orifice and the associated openings of various ducts and what is most likely the clitoris are indicated by I and K, respectively. The round ligament is denoted by G. In Fig. 4, it should be noted that the reproductive tract lies below the urinary bladder (A) and above the rectum (G). The tubular genitalia, including the uterus (B), the body of the uterine tube (C), and the oviduct's terminal infundibulum, with its fimbriae (D), are all suspended within the broad ligament (F in both Fig. 1 and Fig. 4), along with the ovaries (E). As was customary for the particular time period in which it was drawn, **C** shows an extremely mature fetus (A) exhibiting some developmental characteristics more typical of older children or, even, young adults than neonates. This meticulously drawn illustration clearly shows the umbilical cord (B), the amnion (C) and the discoid placenta with its decidual (maternal) and chorionic (fetal) components. The detail in this drawing implies a reasonable understanding of the importance of the placenta and its circulation for fetal nourishment and well-being. Modifications of figures were performed by Howard Wilson and Don Connor.

Puberty and sexual maturity

The onset of puberty

The onset and completion of puberty are potential targets for a variety of reproductive toxicants, and, depending on the toxicant, these events can be hastened or delayed. Puberty in male and female offspring, especially in domestic animals, implies reproductive competence and corresponds to the onset of normal spermatogenesis in the male and reproductive cyclicity in the female. In females of domestic animal species, puberty can be defined by the age at first estrus or ovulation or even the age at which pregnancy can be maintained safely (Evans *et al.*, 1997; Senger, 2007). In the male of most animal species,

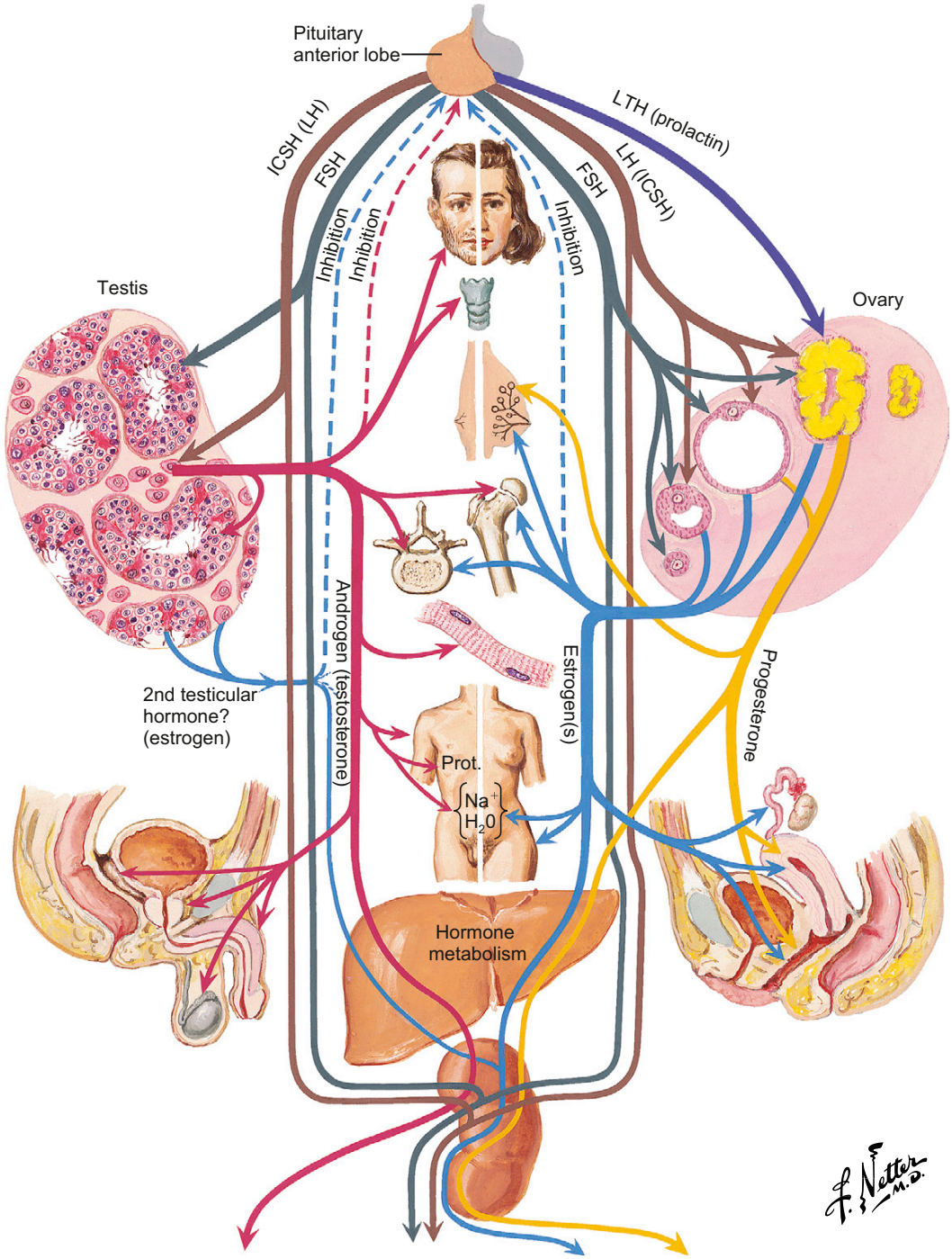

FIGURE 2.3 The basic gonadal steroidogenic pathways, target sites, feedback loops and routes of excretion for the adult male and female human are summarized in this figure. Positive and negative feedback mechanisms involving gonadal steroids help maintain an endocrine environment which is conducive to normal male and female reproductive function. Figure was obtained, with permission, from Netter (1997). Please refer to color plate section.

the age at the time of preputial separation in some species and the acquisition of the ability to ejaculate or the age at the first appearance of spermatozoa in the ejaculate or urine, as well as the production of threshold concentrations of fertile sperm in the ejaculate, have all been used as indicators of puberty (Senger, 2007). Species, nutritional status, environmental and social factors, pheromones and photoperiod in short- or long-day breeders can all influence the age of onset of puberty in animal species (Evans, 2007; Senger, 2007).

In humans, some of the processes by which girls and boys change in appearance and become sexually mature men and women are unique to higher primates. Pubertal development in humans generally takes place in stages and over a longer period of time than in most other animal species (Foster and Gray, 2008; Marshall and Tanner, 1969, 1970). Marshall and Tanner (1969) defined the stages of puberty in girls based on thelarche (i.e., the first stages of breast development), adrenarche, which has been found to be associated with the secretion of androgens (i.e., dehydoepiandrosterone or DHEA and its sulfated conjugate or DHEAS), which induce the growth of pubic hair and alter the composition of sweat gland secretions (Foster and Gray, 2008), and menarche (i.e., the occurrence

of the first menses or sloughing of the endometrial lining in response to cyclic endocrine alterations), which is used by some investigators as a single indicator of puberty. On average, girls begin early breast development by nine or ten years of age, although normally developing girls have been reported to start this process as late as 12 or 13 years of age. Many girls experience menarche between 11.5 and 15.5 years of age and are thought to be sexually mature by the time they reach 14 to 16 years of age (Genuth, 2004b). Similar to puberty in girls, the stages of puberty in boys (Marshall and Tanner, 1970) have been described, at least in part, in terms of the growth of pubic hair related to adrenarche, where, as in girls, adrenarche is associated with phenotypic responses to the androgenic secretions of the zona reticularis portion of the adrenal gland, which develops independent of the maturation of the hypothalamic–pituitary–gonadal axis. The progression of puberty in boys is also evaluated by assessing gonadal and penile growth and development, which unlike adrenarche is dependent on the hypothalamic–pituitary–gonadal axis. On average, boys begin pubertal development by the time they are 10 to 11 years of age, with pubic hair developing between 12 and 16 years of age (Genuth, 2004b). While "full" reproductive function in males is usually achieved by 15 to 17 years of age, this is subject to some variation and is not the same as "maximum reproductive function". In addition to the visual assessment of various physical characteristics related to sexual maturity, the progression of puberty in humans can also be assessed by measurement of serum concentrations of estradiol and testosterone, as well as other estrogens and androgens (Foster and Gray, 2008; Genuth, 2004b).

The endocrinology of puberty

From an endocrine perspective, puberty is associated with maturation of the hypothalamic–pituitary–gonadal axis and the ability of the hypothalamus to release enough GnRH to induce gonadotropin production by the anterior pituitary gland (Evans, 2007; Genuth, 2004b; Senger, 2007). This endocrine milestone is brought about by the postnatal developmental changes which allow the hypothalamus to overcome the negative feedback of testicular androgens and estrogens in males and which facilitate the ovary's ability to produce sufficient estrogens to induce the preovulatory surge of GnRH in females (Evans, 2007; Senger, 2007). Many of the endocrine changes which come into play with the onset of puberty are also involved in the transition from anestrus to the ovulatory season in seasonally polyestrous female animals (Evans, 2007).

Normal male reproductive anatomy and physiology

Developmental perspectives

While the mechanisms of sexual differentiation will be covered in greater detail later in this chapter, it is important to note, as male and, subsequently, female reproductive anatomy and physiology are reviewed, that there is an "undifferentiated" stage during development (Figure 2.4A), where the male fetus is internally and externally indistinguishable from the female fetus. A complex set of structural modifications (Figures 2.5A and 2.5B) result in what is seen internally, with respect to the testes and excurrent duct system (Figure 2.4A), as well as externally for penile and scrotal morphology (Figure 2.4B).

Reproductive anatomy of the male

Anatomical structures associated with reproduction in the male usually include, especially in mammals, paired testes (i.e., male gonads) positioned outside the abdominal cavity in most species; an excurrent duct system (i.e., efferent ductules, paired epididymidies, ducti deferens and urethra); accessory sex glands (i.e., ampullae, seminal vesicles, prostate and bulbourethral glands); a scrotum and its associated thermoregulatory functions to protect the testes from mechanical and thermal insult; and some form of copulatory organ or penis with a mechanism for protrusion, erection, emission of glandular secretions and sperm into the urethra and ejaculation of semen from the urethra at the time of orgasm (Figure 2.2A). The primary functions of the testis (testicle) are spermatogenesis or production of male gametes (sperm or spermatozoa) and steroidogenesis (production of androgens and estrogens). Unlike the female in which oogonia are no longer replicating and the full complement of potential oocytes is present at birth, spermatogonia are proliferating and differentiating into spermatozoa continuously, and the testis is organized in such a way as to maximize sperm production (Evans, 2007; Foster and Gray, 2008; Senger, 2007). Figure 2.2A clearly shows the primary anatomical components of the male reproductive tract, and, while the names and understanding of the underlying cellular and molecular processes taking place in these tissues have changed over the last 200 years, the appearance of these structures and how they are presented in anatomical illustrations has essentially remained unchanged.

Testicular structure

Taking a closer look at the human testis, it is evident that the testis is divided into lobules of parenchyma consisting of tubular and interstitial compartments (Evans, 2007; Netter, 1997; Senger, 2007). The structural and functional units within the tubular compartment are the seminiferous tubules (Figures 2.6A and 2.6B), which, depending on the species, comprise approximately 80% of the adult testis (Genuth, 2004b). As shown in Figure 2.6A, seminiferous tubules form highly convoluted loops (tubulus contortus) which begin and end with straight portions (tubulus rectus) that connect to the rete tubules (Genuth, 2004b; Netter, 1997; Senger, 2007). In some species, such as the human, the rete tubules coalesce in a fibrous region of the testis referred to as the mediastinum, which joins with septal projections of the tunica albuginea, part of the testicular capsule. The rete tubules join with the efferent ductules, which attach to the epididymidis, which leads into the ductus deferens or vas deferens.

Within the seminiferous tubules are germ cells at various stages of differentiation and Sertoli cells, which provide germ cells with structural support and nutrients, as well as regulatory and paracrine factors (Foster and Gray, 2008) (Figure 2.6B). Tight junctions (junctional complexes) between adjacent Sertoli cells divide the seminiferous epithelium into basal and adluminal compartments, with Sertoli cells anchored to the basement membrane and surrounding the developing populations of germ cells (Evans, 2007; Foster

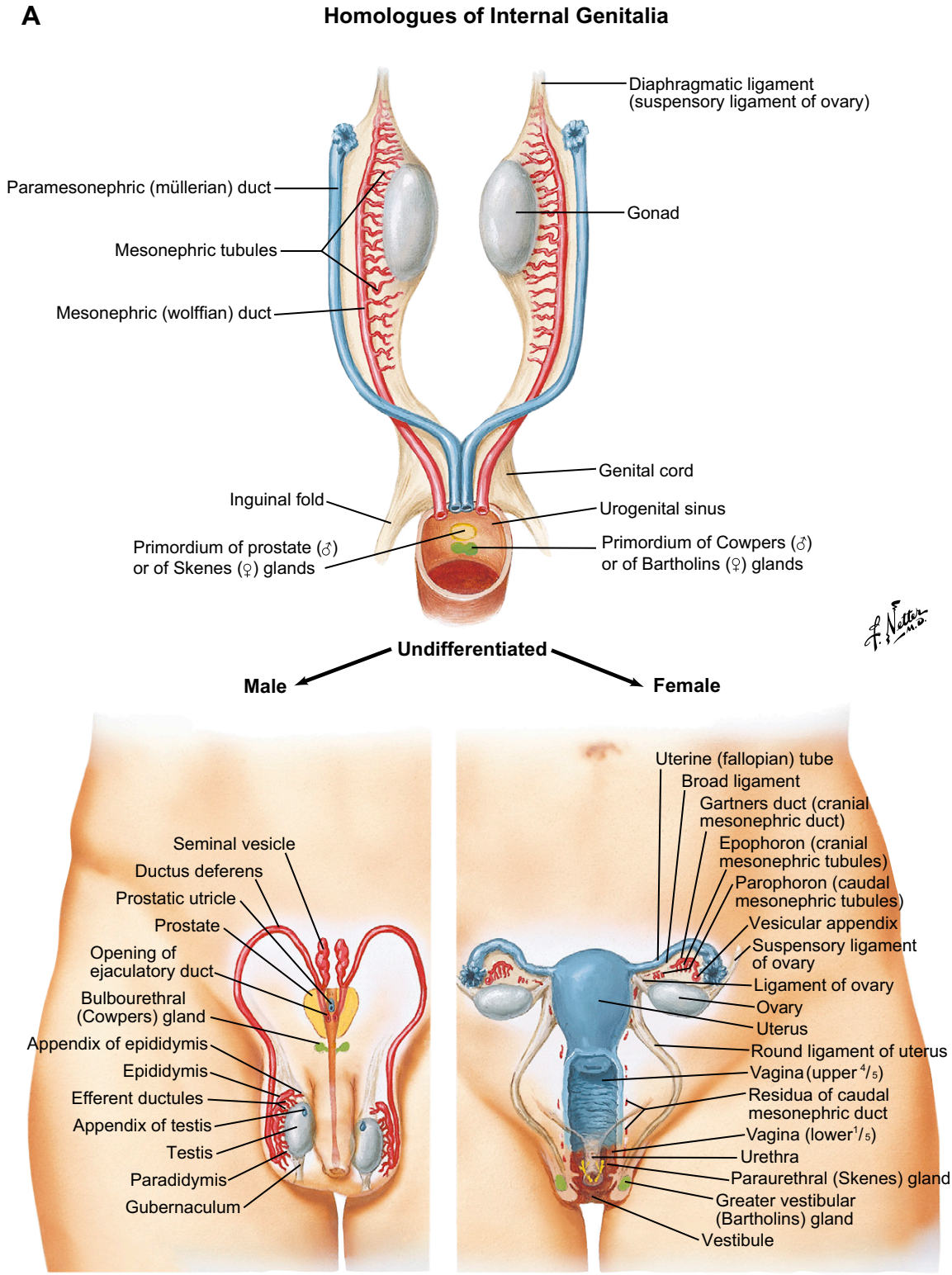

FIGURE 2.4 The "undifferentiated" stage observed in the fetus, regardless of genotypic sex, early in gestation prior to gonadal sexual differentiation, as well as the gonads, internal genitalia and other associated anatomical structures of the sexually mature male and female are shown in **A**. **B** (page 14) illustrates the standard sequence of events in the development of the external genitalia of men and women, as well as other mammalian species. The failure of the urethral groove to close at any point during this sequence results in various degrees of hypospadias, which is a relatively common congenital birth defect in male offspring and one which has been induced in laboratory species by prenatal exposure to a number of xenobiotics. Figures were obtained, with permission, from Netter (1997). Please refer to color plate section.

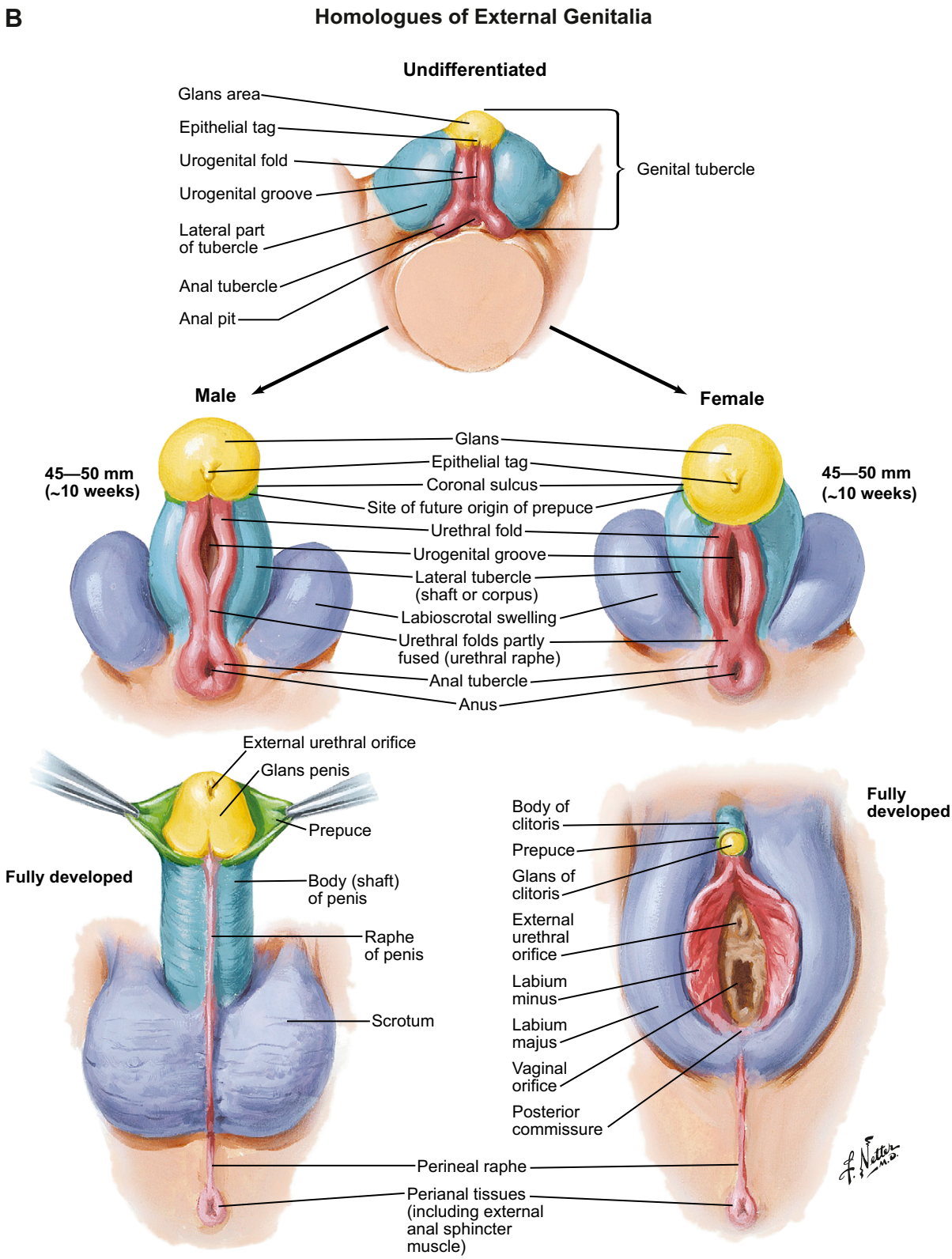

FIGURE 2.4—Cont'd

and Gray, 2008; Genuth, 2004b; Senger, 2007). The seminiferous tubules are surrounded by peritubular myoid cells which participate in important cell–cell interactions with Sertoli cells, the junctional complexes of which form the "blood–testis barrier" or "Sertoli cell barrier" to prevent free exchange of large proteins and some xenobiotics between the blood and the fluid within the seminiferous tubules (Hess and França, 2005; Senger, 2007). It should be noted from Figure 2.6B that, as expected, the appearance of the seminiferous tubules changes as male offspring mature postnatally.

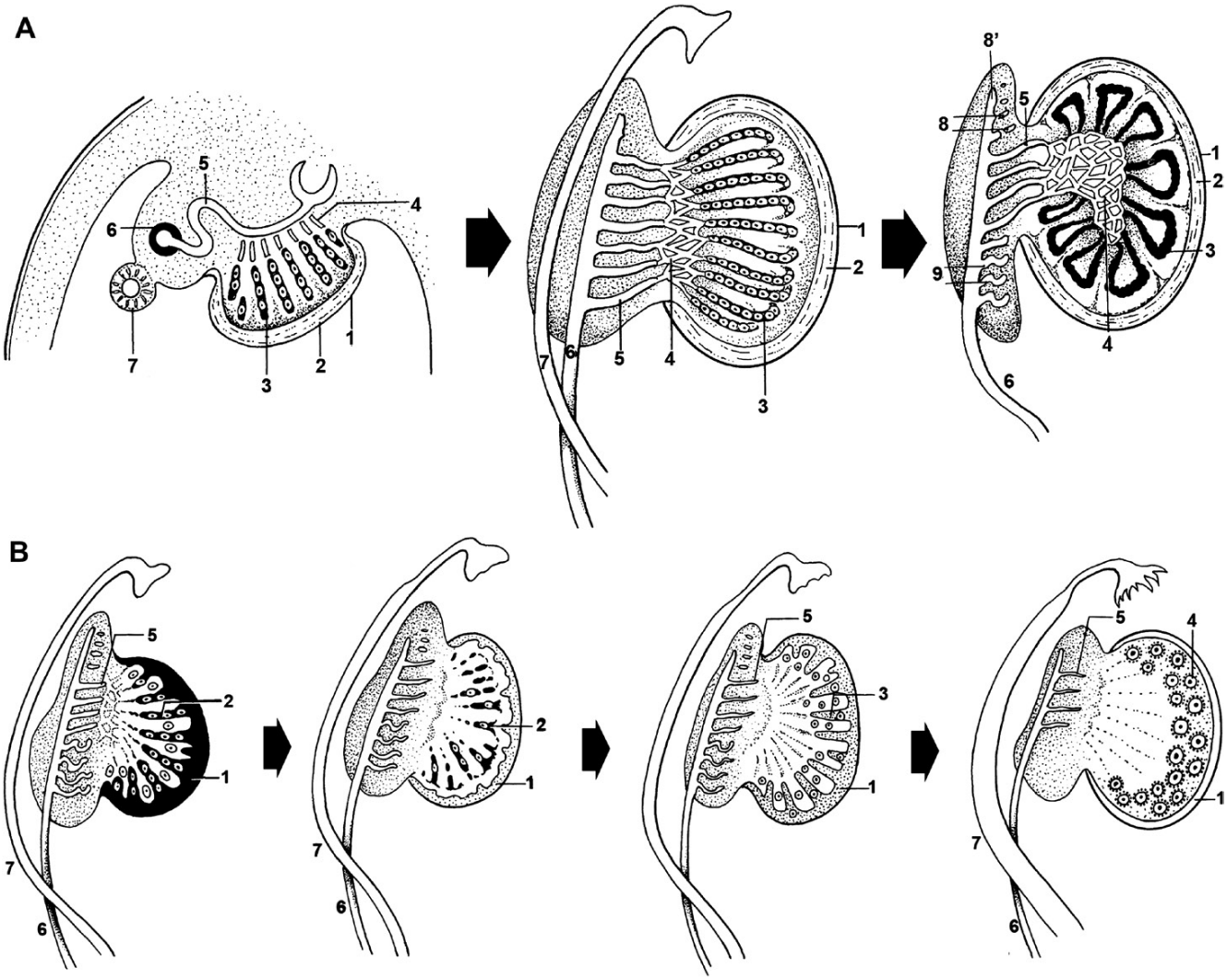

FIGURE 2.5 The initial stages in the development of the testis and the formation of the excurrent duct system are shown in **A**. The initial formation of the tunica albuginea isolates the epithelial cords from the surface epithelium, and the epithelial cords, rete testis and mesonephric tubules (also referred to as the mesonephric ductules or mesonephric duct system) subsequently interconnect. The epithelial cords (sex cords) will eventually become the seminiferous tubules, and the mesonephric ductules will be incorporated into the formation of the excurrent duct system. (1) Celomic epithelium; (2) tunica albuginea; (3) epithelial cords (future seminiferous tubules); (4) rete testis; (5) mesonephric tubules (later efferent ductules); (6) mesonephric duct (future epididymis (proximal portion contiguous with mesonephric tubules and ductus deferens (distal portion)); (7) paramesonephric duct; (8) cranial remnant of mesonephric duct system (aberrant ductules); (8′) remnant of mesonephric duct (appendix of epididymis); and (9) caudal remnant of mesonephric duct (paradidymis). The initial stages in the development of the ovary and the formation of paramesonephric ducts are shown in **B**. The epithelial cords (sex cords) penetrate and then regress within the developing ovary, eventually fragmenting and organizing into cell clusters which consist of a single oocyte surrounded by a layer of granulosa cells (primordial follicles). The paramesonephric ducts undergo further development and differentiation, and the mesonephric duct system begins to regress. (1) Celomic epithelium; (2) epithelial cords which initially penetrate then regress and fragment; (3) early formation of future cortical region; (4) primordial follicles; (5) regressing mesonephric tubules; (6) mesonephric duct which will eventually regress; and (7) paramesonephric duct which will undergo further development and differentiation into the major female tubular genitalia. This figure was adapted, with permission, from Gupta (2007). Modifications were courtesy of Don Connor and Howard Wilson.

Within the interstitial compartment, the primary cellular components are the Leydig or interstitial cells, and capillaries, lymphatic vessels and connective tissue are also present in this portion of the testicular parenchyma (Evans, 2007; Senger, 2007). The Leydig cells are homologous to the theca interna cells in the ovary and produce testosterone (also estrogen in some species). There are species differences with respect to the abundance of Leydig cells in the interstitium, and these differences are important to recognize when reporting Leydig or interstitial cell hyperplasia in response to toxicant exposure. It should also be noted that Leydig and, to a lesser extent, Sertoli cells contain enzymes involved in xenobiotic biotransformation, and the synthesis of toxic metabolites can actually occur within the testis, in close proximity to the target cells for a given reproductive toxicant.

Excurrent duct system

The excurrent duct system for each testis consists of the efferent ductules, the epididymal duct and the ductus deferens. This duct system functions to conduct spermatozoa, rete fluid and some testicular secretory products away from the testis and eventually into the pelvic urethra (Senger, 2007). The

reabsorption of fluid by a species-variable number of efferent ductules is essential for normal testicular function (Hess, 2003; O'Donnell *et al.*, 2001), and these tubules terminate by joining a single highly coiled epididymal duct, commonly referred to as the epididymidis or epididymis. Depending on the species, the epididymidis is generally subdivided into the initial segment, head (caput), body (corpus) and tail (cauda), with the various portions sometimes being further subdivided (França *et al.*, 2005; Senger, 2007). The primary functions of the epididymidis are transport and sustenance of sperm; reabsorption and secretion of fluid (initial segment and head, respectively); spermatozoal acquisition of motility and fertile potential (i.e., sperm maturation); recognition and elimination of defective spermatozoa; sperm storage prior to ejaculation; and secretory contributions to the seminal fluid (Evans, 2007; Sutovsky *et al.*, 2001). The epididymal transit time varies somewhat with species, but is generally approximately 7 to 14 days in length, depending on several factors, including ejaculation frequency. The ductus deferens conducts spermatozoa matured in the epididymidis to the pelvic urethra which helps to form the penis.

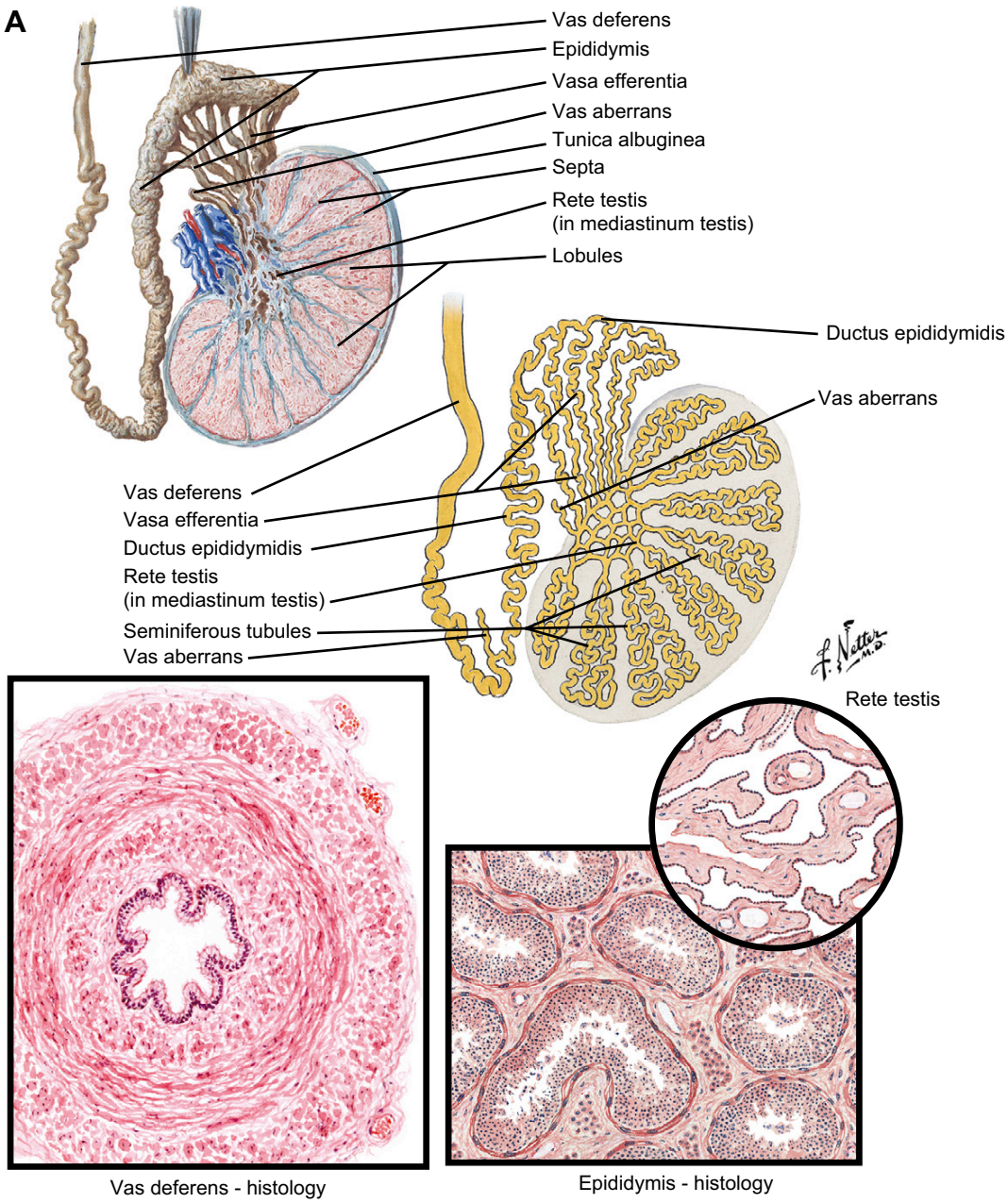

FIGURE 2.6 The structural relationships between the tunica albuginea, septa, lobules of testicular parenchyma, the mediastinum testis and the excurrent duct system within the testes of humans are shown in **A**. **A** also illustrates the sequential transport of sperm through the loops of the seminiferous tubules, rete testis and the excurrent duct system, which includes the efferent ductules (vasa efferentia), epididymidis (epididymis) and ductus deferens (vas deferens) and shows cross-sections of the rete testis, epididymis and ductus deferens within the mature human testis. The structural and functional units within the tubular compartment are the seminiferous tubules, and the complex nature of the association between Sertoli cells and developing germ cells within the seminiferous epithelium of the human testis, including during various stages of sexual maturity, are shown in **B**. Figures were obtained and modified, with permission, from Netter (1997).

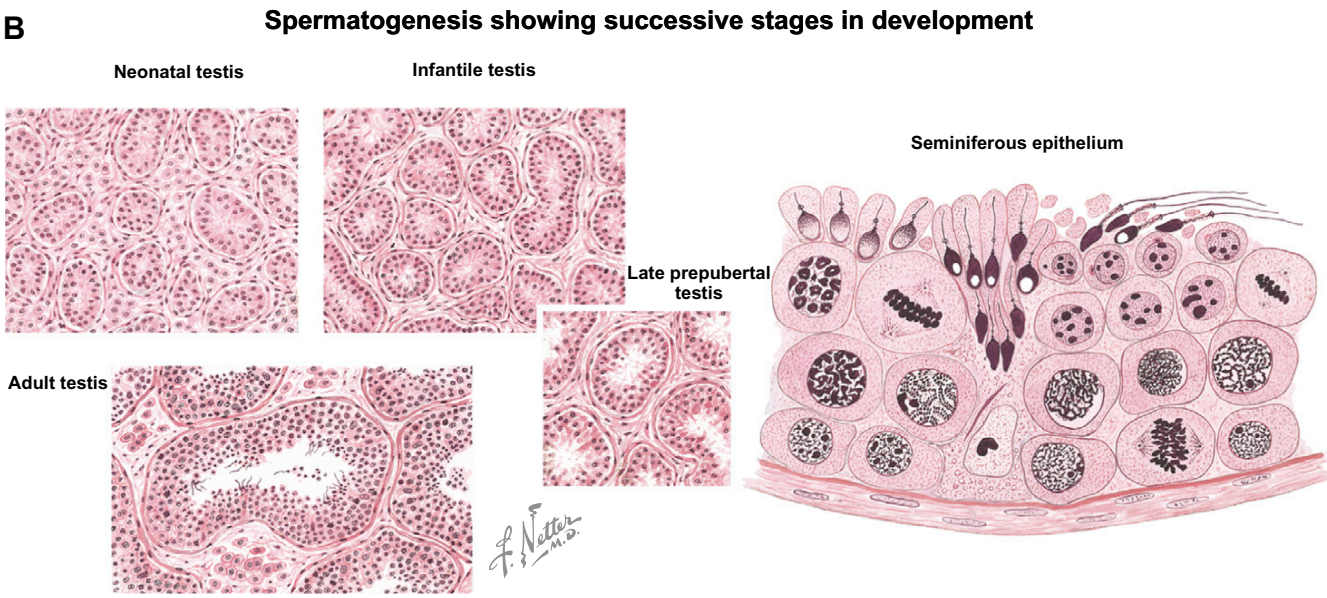

FIGURE 2.6—Cont'd

Accessory sex glands

There are a number of accessory sex glands (the complement of which varies with species) that contribute to the composition of the seminal fluid in mammals. In humans, these glands include the ampullae, seminal vesicles (vesicular glands), prostate and bulbourethral glands (Haschek *et al.*, 2010; Senger, 2007) (Figures 2.2A and 2.7). Laboratory rodents (i.e., mice and rats) have an additional gland referred to as the preputial gland, which appears to have a role in the production of pheromone (Haschek *et al.*, 2010). These accessory sex glands in the male are generally considered to be androgen dependent, with the conversion of testosterone to DHT occurring in the prostate and seminal vesicles of many species (Evans, 2007; Senger, 2007). The weights of the accessory sex glands can be used as an indirect measure of testosterone concentrations or exposure to antiandrogens (Foster and Gray, 2008; Haschek *et al.*, 2010). The human prostate gland is particularly susceptible to the development of benign prostatic hypertrophy (BPH) and various neoplasias, so that familiarity with its internal and external structure can be very useful when evaluating xenobiotic-induced alterations (Figure 2.7).

External genitalia

The external genitalia of the male consist of the copulatory organ or penis, the prepuce or foreskin, which protects the penis from environmental and mechanical injury, and the scrotum for testes positioned outside the abdominal cavity. In humans, the foreskin is frequently removed shortly after birth by circumcision. Penile structure is extremely species variable, with some species even having a special penile bone (i.e., os penis), but the shaft of the penis generally consists of erectile tissue (corpus cavernosum and corpus spongiosum) which surrounds the pelvic urethra. The development of the external genitalia follows a standard sequence of events, and the failure of the urethral groove to close at any point during this sequence results in various degrees of hypospadias (Figure 2.4B). As shown in Figure 2.4B, the glans penis is homologous to the female clitoris, and stimulation of the glans is the primary factor involved in the initiation of ejaculation (Netter, 1997; Senger, 2007). The scrotum protects the testes from mechanical injury and, in conjunction with the tunica dartos, cremaster muscle and pampiniform plexus, plays a major thermoregulatory role with respect to temperature-sensitive, testicular spermatogenesis (Senger, 2007). In some species of wildlife (e.g., elephants and marine mammals), the testes are positioned intra-abdominally.

Spermatogenesis

Spermatozoa are highly specialized haploid cells equipped with a self-powered flagellum to facilitate motility, as well as an acrosome to mediate penetration of the zona pellucida. Spermatogenesis takes place within the seminiferous tubules and consists of all the changes germ cells undergo in the seminiferous epithelium in order to produce adequate numbers of viable spermatozoa each day and to continuously replace spermatogonial stem cells (Evans, 2007; Foster and Gray, 2008). Spermatogenesis provides for genetic diversity and ensures that germ cells are in an immunologically favored site (Senger, 2007). The duration of spermatogenesis varies with species but generally ranges between 4 and 8 weeks (approximately 30 to 60 days) in domestic and laboratory animals and is approximately 75 days (almost 11 weeks) in humans. It is important to keep in mind the durations of spermatogenesis and epididymal sperm transport in a given species, as well as the normal, species-specific number of spermatozoa produced daily by the testes, when determining the period of toxicant exposure relative to the appearance of abnormal spermatozoa in an ejaculate and when assessing the severity and reversibility of toxicant-induced damage to sperm precursors within the testes (Evans, 2007).

Spermatogenesis can be subdivided into three phases or stages referred to as "proliferation", "meiosis" and "differentiation". During each of these phases, sperm precursors or male germ cells (spermatogonia, spermatocytes or spermatids) undergo specific, stepwise changes as they develop

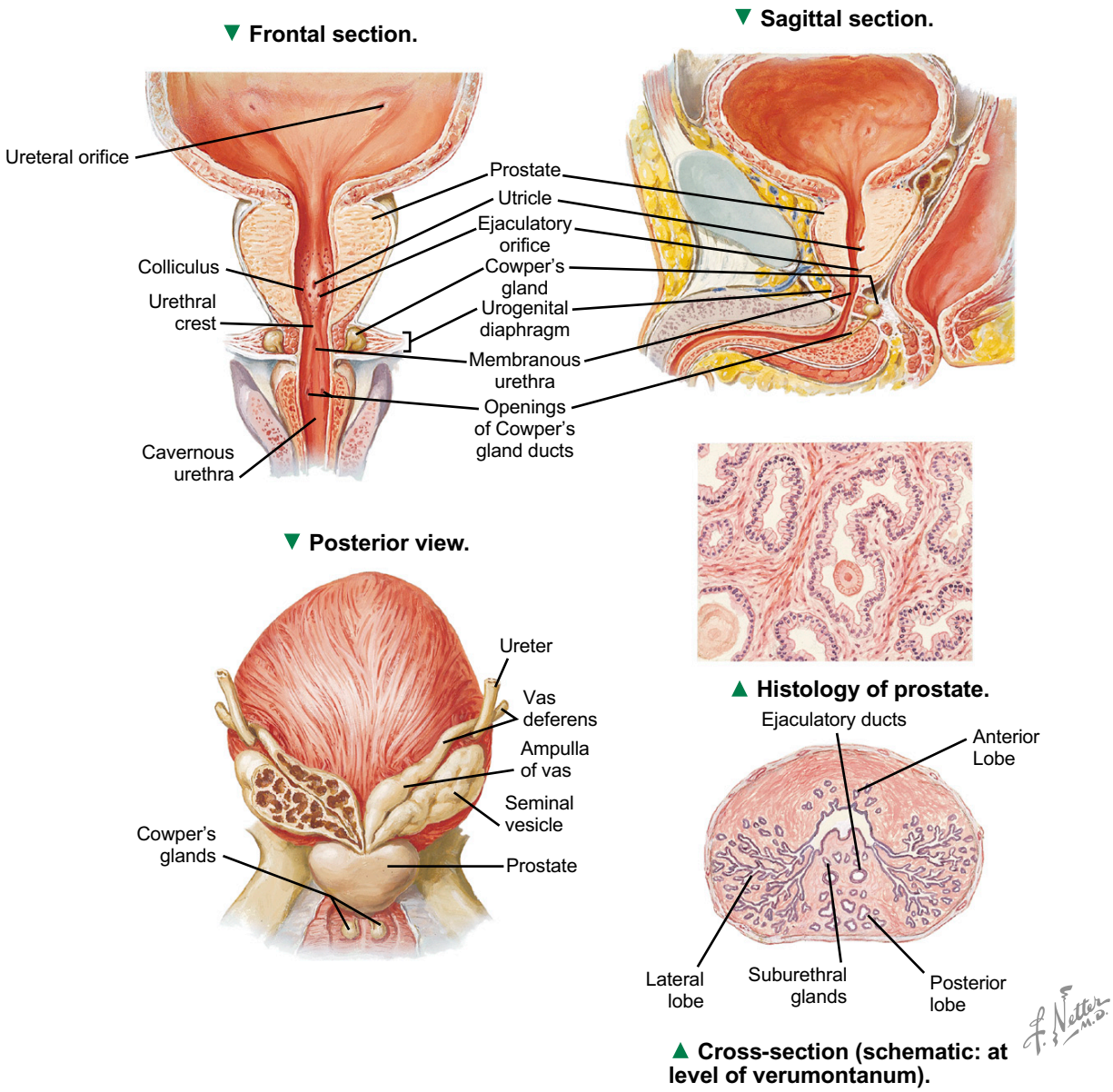

FIGURE 2.7 The anatomical relationships between the accessory sex glands in sexually mature men and the histological appearance of the human prostate gland are shown. Figure was obtained, with permission, from Netter (1997).

into spermatozoa which will eventually be released into the excurrent duct system. Each of these phases involves a different type of germ cell undergoing a different developmental process, and, as such, these phases have the potential to differ in their susceptibility to the mechanisms of action of various reproductive toxicants (Evans, 2007; Foster and Gray, 2008).

Proliferation (mitosis or spermatocytogenesis)

The "proliferation" phase of spermatogenesis has also been referred to as "mitosis" or "spermatocytogenesis" and occurs within the basal compartment of the seminiferous tubule. Proliferation denotes all of the mitotic divisions involving spermatogonia (Foster and Gray, 2008; Senger, 2007). A large number of B-spermatogonia result from the mitoses of several generations of spermatogonia (e.g., A_1, A_2, A_3, A_4 and I; some species variations in nomenclature) (Genuth, 2004b; Senger, 2007). Stem cell renewal is accomplished during proliferation by the reversion of some spermatogonia to more primitive germ cells (Senger, 2007). Germ cell mitosis during spermatogenesis ends with the transformation of B-spermatogonia into primary spermatocytes, and this process is particularly susceptible to toxicants, such as chemotherapeutic agents and radiation, which target rapidly dividing cells (Evans, 2007).

Meiosis

"Meiosis" takes place within the adluminal compartment of the seminiferous tubules and involves the participation of primary and secondary spermatocytes in a total of two

meiotic divisions. The chromosomal reduplication, synapsis and cross-over, as well as cellular division and separation, which occur during this phase of spermatogenesis, are extremely complex and guarantee genetic diversity (Genuth, 2004b; Senger, 2007). The meiosis phase of spermatogenesis is considered by some to be most susceptible to toxic insult and ends with the production of haploid round spermatids.

Differentiation (spermiogenesis)

Spermatozoa have been aptly characterized as "sophisticated, self-propelled packages of DNA and enzymes" (Senger, 2007). "Differentiation" or "spermiogenesis" involves all the changes occurring within the adluminal compartment, which transform round spermatids into spermatozoa possessing an acrosome for penetration of the zona pellucida and a tail or flagellum to facilitate motility (Genuth, 2004b). Differentiation can be subdivided into the "Golgi", "cap", "acrosomal" and "maturation" phases, which correspond respectively to acrosomal vesicle formation; spreading of the acrosomal vesicle over the nucleus; elongation of the nucleus and cytoplasm; and final assembly involving the formation of the postnuclear cap organization of the tail components (Senger, 2007). Following the nuclear and cytoplasmic reorganization which characterizes the changes to germ cells during spermiogenesis, differentiated spermatozoa are released from Sertoli cells into the lumen of the seminiferous tubules by a process referred to as "spermiation". The complex signaling pathways and genomic imprinting involved in regulating the differentiation of round spermatids into spermatozoa are potential targets for endocrine disrupting chemicals (EDCs) or endocrine disruptors (Evans, 2007; Foster and Gray, 2008).

The cycle of the seminiferous epithelium

In most sexually mature mammals, spermatozoa are produced continuously, with the entry of germ cells into the proliferation phase of spermatogenesis occurring in a coordinated cyclic manner (Foster and Gray, 2008; Genuth, 2004b). Spermatogonia A in a given region of the seminiferous tubule commit to proliferate in a synchronous manner, with cohorts of their progeny germ cells (cellular generations) connected by intercellular bridges and developing and differentiating in unison. Including spermatogonia A, four or five generations or concentric layers of sperm precursors are present in each cross-section of the seminiferous tubules (Figure 2.6B). The cycle of the seminiferous epithelium in most mammals is characterized by germ cells in each spermatogenic phase associating with contiguous generations in a repeatable pattern of specific cellular associations or "stages" (Foster and Gray, 2008; França *et al.*, 2005). There is generally only one stage per seminiferous tubular cross-section section in subprimates, and each stage transitions into the next at predictable intervals (Senger, 2007). At any given point along a seminiferous tubule, the entire cycle of the seminiferous epithelium occurs over a set time interval closely associated with the spermatogonial turnover rate for that particular mammalian species. The number and durations of the various stages of the cycle of the seminiferous epithelium vary with species, and various classification schemes have been used, based on the morphological characteristics of the spermatid nucleus or the development of the acrosomic system. In subprimates, sequential stages are arranged along the length of the seminiferous tubule in consecutive order, forming a "spermatogenic wave" (Haschek *et al.*, 2010; Senger, 2007). The progeny of one spermatogonium A will progress through approximately 4.5 cycles of the seminiferous epithelium before being released into the lumen of the seminiferous tubule and progressing through the rete testis into the excurrent duct system. An understanding of the cycle of the seminiferous epithelium is very useful for the evaluation of the effects of xenobiotics on spermatogenesis and for the determination of the populations of germ cells most susceptible to a given toxicant.

Male reproductive physiology

Gonadal steroid synthesis in the testes

The endocrine events which regulate spermatogenesis and sexual behavior in males are very distinct from those which take place in females. The primary gonadal steroids produced by the testes are androgens, testosterone and DHT, which are also produced from testosterone in selected nongonadal tissues, and estrogens (primarily estradiol in most species), which are now recognized as playing essential roles in male reproductive development and function (Hess, 2003; O'Donnell *et al.*, 2001). Leydig cells in the interstitium synthesize pregnenolone and then progesterone from cholesterol and convert progesterone to testosterone under the influence of LH (Genuth, 2004b; Senger, 2007). The site of estrogen synthesis (i.e., aromatase activity) varies with the age and species of animal. In the male fetus, postnatal immature male and, in some species, the adult male, Sertoli cells within the seminiferous tubules play a major role in the aromatase-mediated conversion of testosterone to estradiol under the influence of FSH. In many mammals, however, Leydig cells in the fetal testis and, especially, the postnatal immature testis gradually begin to synthesize estrogens, and, at sexual maturity, a major portion of the estrogens in these species are produced by aromatase activity in the Leydig cells, under the influence of LH rather than FSH (Hess, 2003; O'Donnell, 2001; Payne, 2007). More recently, germ cells have been identified as another potential source of estrogen in the testis, and it is possible that germ cell-derived estrogens play major roles in regulating male reproductive function (Hess, 2003).

Endocrine regulation of spermatogenesis

The basic gonadal steroidogenic pathways, target sites, feedback loops and routes of excretion for the adult male human are summarized in Figure 2.3. While the female hypothalamus has both fully developed tonic and surge centers for GnRH release (especially prior to ovulation), the hypothalamic GnRH surge center in the male is diminished, and the anterior pituitary gland of the male does not experience surges in GnRH stimulation. This sex-specific alteration in the hypothalamus facilitates the normal endocrine milieu which maintains continuous spermatogenesis and stimulates normal sexual behavior. The tonic pulsatile release of GnRH induces the anterior pituitary to produce pulses of LH and FSH several times during the day and facilitates adequate LH-dependent testosterone production and, depending on

the species, normal FSH-dependent Sertoli function, both of which are essential for spermatogenesis to occur continuously in the seminiferous tubules. In some species, FSH is primarily required for the onset of puberty and the initiation of spermatogenesis, with many of the functions of FSH in the immature male being taken over by testosterone in the sexually mature animal (Evans, 2007). Testosterone stimulates Sertoli cells to produce several androgen-regulated proteins (including androgen-binding protein) which are required for spermatogenesis. Estrogens are required for various aspects of the normal development and function of Sertoli cells and germ cells within the seminiferous tubules. Xenobiotics which mimic or inhibit the actions of estradiol within the testis can disrupt normal spermatogenesis.

Positive and negative feedback loops involved in male reproduction

Positive and negative feedback mechanisms involving gonadal steroids help maintain an endocrine environment which is conducive to normal male reproductive function (Figure 2.3). In addition to these feedback loops, the Sertoli cell can produce activin and inhibin which respectively increase and decrease the secretion of FSH by gonadotropes and, in some species, GnRH release from the hypothalamus (Haschek *et al.*, 2010). Testosterone, DHT and estradiol all provide negative feedback to the hypothalamus with respect to GnRH release, and testosterone can also directly inhibit LH secretion by gonadotropes (Haschek *et al.*, 2010; Senger, 2007). Xenoestrogens and xenoandrogens have the potential to disturb the hypothalamic–pituitary–gonadal axis (O'Donnell *et al.*, 2001). Antiandrogens and a variety of other xenobiotics can interfere with this feedback loop, resulting in excessive secretion of LH and Leydig or interstitial cell hyperplasia (Evans, 2007; Foster and Gray, 2008).

Epididymal and accessory sex gland function

Epididymal development and function are dependent on the proper balance of androgenic and estrogenic stimulation and are required for normal male reproductive function and fertility. The accessory sex glands are considered to be primarily androgen dependent, and the secretions of these glands, as well as those of the epididymidis, are important components of seminal fluid. Conversion of testosterone to DHT can generally occur in the epididymidis, prostate and seminal vesicles. Hormonally active xenobiotics, which alter the normal endocrine events associated with epididymal and accessory gland development and function, can have adverse effects on male fertility (Evans, 2007).

Sexual behavior, erection, emission and ejaculation

Sexual behavior is mediated by estradiol in postnatal males and females. The conversion of the steadily produced testosterone in the male to estradiol in the brain (plus the effects of estrogens of testicular origin) results in the male being sexually receptive most of the time (Evans, 2007; Senger, 2007). Adequate libido and sexual receptivity, as well as adequate concentrations of testosterone, are necessary for erection of the penis, which is required for intromission during copulation (Sikka, *et al.*, 2005). Olfactory (detection of pheromones), auditory and visual stimuli play roles in facilitating cholinergic and NANC (non-adrenergic/non-cholinergic) parasympathetic neuron-mediated penile erection, which, especially in men and stallions, requires a significant amount of nitric oxide-associated vasodilation and vascular engorgement. During copulation, the events which lead to emission of the secretions of the accessory sex glands and sperm (i.e., semen) into the urethra and the ejaculation of semen from the urethra at the time of orgasm generally involve tactile stimuli to the glans penis, stimulation by sympathetic neurons and spinal reflexes.

Normal female reproductive anatomy and physiology

Developmental perspectives

Similar to the male, there is an "undifferentiated" stage during development (Figure 2.4A), where the female fetus is internally and externally indistinguishable from the male fetus. What is eventually observed internally (Figure 2.4A) as well as externally (Figure 2.4B) in the female is due to a complex set of structural modifications (Figure 2.5B), resulting in the formation of the ovary and the internal tubular genitalia.

Reproductive anatomy of the female

Although there are some distinct morphological differences between species (e.g., simplex uterus in primates, duplex cervices in rabbits), the female reproductive tract, as shown for humans in Figure 2.2B, generally consists of paired ovaries, the "tubular genitalia", which include the paired oviducts (uterine tubes), the contiguous uterus, cervix, vagina, vestibule and vulva. In species other than humans and other higher primates, there are also separate uterine horns of varying lengths and degrees of curvature, which connect with the uterus (Evans, 2007; Senger, 2007). As in the male, the organs involved in female reproductive function have been well recognized for over 200 years and are physiologically and morphologically dynamic. They function to produce the oocyte, facilitate its fertilization, provide an environment for embryonic and fetal development, and transport the fetus from the maternal to the external environment. Variations in size, appearance, location and function of the female reproductive organs depend on the endocrine milieu dictated by the effects of sexual maturation, stage of the estrous or menstrual cycle, gestational hormone production of maternal, fetal and/or placental origin, exposure to exogenous hormonally active agents or HAAs (sometimes used interchangeably with EDCs or endocrine disruptors) and seasonal influences (Evans, 2007; Foster and Gray, 2008; Netter, 1997; Senger, 2007).

The primary functions of the ovary are oogenesis or production of female gametes (oocytes or ova) and steroidogenesis (production of estrogens and progesterone). The ovaries of most domestic mammals consist of a peripheral parenchymatous zone (cortex), containing various stages of follicular and luteal gland development and a central vascular zone (medulla), comprised of collagenous connective tissue rich in blood vessels (Evans, 2007; Foster and Gray, 2008; Genuth, 2004b; Senger, 2007). The structural and functional unit of the ovary is the

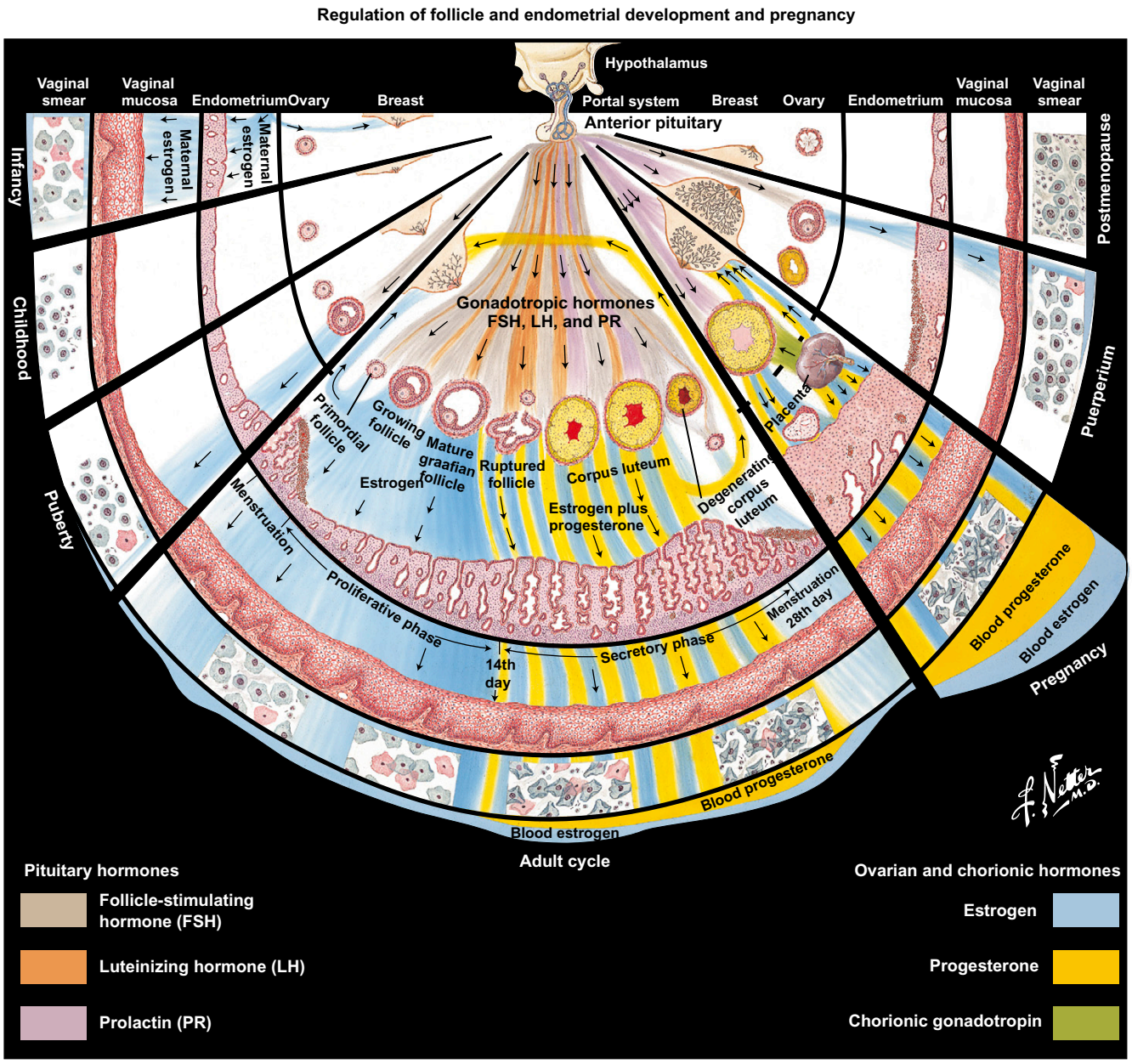

FIGURE 2.8 In humans, chemical exposures can take place over an entire lifetime, and early xenobiotic exposures have the potential to affect reproductive events occurring later in life. This figure clearly and comprehensively summarizes all of the anatomical and physiological reproductive changes which can take place in women's lives between infancy and menopause, including those associated with puberty and the various stages of the menstrual cycle, as well as periods of pregnancy and lactation. The transition between the various aspects of a woman's reproductive activity involves alterations in anterior pituitary hormone secretion and structural and functional modifications in the ovaries, endometrium, vaginal epithelium and the mammary glands. Figure was obtained, with permission, from Netter (1997). Please refer to color plate section.

follicle (Figure 2.8). Follicles are classified as primordial, primary (some become atretic), secondary and tertiary (i.e., antral) follicles based on their stage of development (Evans, 2007).

A primary oocyte surrounded by a single, flattened cell layer is a primordial follicle. A basal lamina separates the single layer of what will become granulosa cells from the adjacent stromal tissue which eventually develops into the theca cells (theca interna and theca externa). The granulosa cells are homologous to the Sertoli cells in the testis, and the theca interna cells are the female equivalent of the Leydig cells (Evans, 2007; Senger, 2007). Following the appropriate endocrine stimulation, primordial follicles are recruited to undergo possible further differentiation into estrogen-producing antral (i.e., tertiary) follicles and ultimately ovulation, which results in the release of a secondary oocyte (primary oocyte in dogs) and formation of a corpus luteum (CL) which produces progesterone.

Female reproductive physiology

Females are born with a finite pool of primordial follicles (up to hundreds of thousands), and reproductive cyclicity (i.e., estrous or menstrual cycles) provides females with repeated opportunities for the establishment of pregnancy.

The majority of mammalian species (subprimates) have estrous cycles, which reflect the physiologic changes occurring between successive ovulations and/or periods of sexual receptivity (estrus) (Senger, 2007). Humans and non-human primates experience menstrual rather than estrous cycles and do not have defined periods of sexual receptivity (i.e., estrus). As illustrated in Figure 2.8, unlike the estrous cycles in subprimates, the reproductive cycle in menstruating animals is divided into phases (i.e., menses, proliferative and secretory phases), which are defined based on the physiological state of the uterine endometrium, rather than on the predominant ovarian structures (i.e., estrous cycles) (Genuth, 2004b; Netter, 1997; Senger, 2007).

The estrous cycle

The follicular and luteal phases of the estrous cycle describe the predominant ovarian structures and the corresponding gonadal steroid concentrations which result from the follicular secretion of estrogens or the luteal secretion of progesterone, respectively (Evans, 2007; Senger, 2007). Both the follicular and luteal phases can generally be further subdivided into two stages each, proestrus and estrus (sexual receptivity) for the follicular phase and metestrus and diestrus (sexual non-receptivity) for the luteal phase. Proestrus represents the period of transition from the diestrous dominance of progesterone to the dominance of estrogens during estrus, while metestrus represents the opposite shift in the endocrine milieu (estrogen dominance to progesterone dominance).

The menstrual cycle

The reader is directed to Figure 2.8 in order to best understand the sequence of the morphological and endocrine events which take place during the menstrual cycle in women, which is generally 28 days in duration. As mentioned earlier, the menstrual cycle is defined in terms of phases corresponding to events occurring within the endometrium, rather than within the ovary; however, an effort will be made here to discuss also ovarian events taking place at the same time as the endometrial changes so one can understand the correlations between the estrous and menstrual cycles. At the beginning of menses, follicles develop under the influence of FSH (i.e., follicular phase begins), with minimal LH secretion, thereby reflecting anterior pituitary sensitivity to GnRH (Genuth, 2004b). As tertiary follicles develop, more estrogens are produced, and the endometrium enters the proliferative stage. Estrogens provide negative feedback for FSH secretion and positive feedback for LH release by the anterior pituitary. The increasing amount of LH and decreasing FSH results in ovulation about midway through the cycle (i.e., day 14), and the follicle begins to undergo luteinization and forms corpus luteum, which produces progesterone, as well as estrogen (i.e., luteal phase begins) (Genuth, 2004b). The secretory phase of the endometrium begins as the corpus luteum forms and secretes progesterone and estrogens. In response to feedback loops involving this secreted progesterone and estrogens, the relative proportions of FSH and LH secreted by the anterior pituitary change, with subtle decreases in the amounts of progesterone and estrogens produced by the corpus luteum observed. During the late secretory phase of the endometrium, the absence of a conceptus in the uterus results in the regression of the corpus luteum (i.e., luteolysis), a precipitous decrease in the secretion of progesterone and estrogens, the local production and subsequent release of leukotrienes and prostaglandins within the endometrium, and the subsequent cascade of vascular events which result in the sloughing of the endometrium accompanied by bleeding, which are characteristic of menses (Figure 2.8) (Genuth, 2004b; Netter, 1997).

Follicular development

The general sequence of endocrine and morphologic changes occurring during the estrous and menstrual cycles involves a variety of positive and negative feedback loops affecting the hypothalamic–pituitary–gonadal axis and leads to the development of antral follicles, the primary source of estrogens, and, eventually, the formation of corpora lutea, which produce progesterone (Figures 2.3 and 2.8). When females are exhibiting reproductive cyclicity, there are cyclic alterations in the pattern of hypothalamic GnRH secretion from the tonic and surge centers, which interact with the anterior pituitary to influence the relative amounts of FSH and LH secreted by anterior pituitary gonadotropes. Over the course of sequential ovulatory cycles, many (up to several hundred or more, depending on the species) primordial follicles leave the reserve pool in a cyclic fashion (under the influence of FSH) and enter the active pool of follicles (primary follicles) undergoing growth and differentiation (folliculogenesis) and eventually atresia or ovulation (Evans *et al.*, 1997; Senger, 2007). The oocyte in the developing follicle grows in size, the zona pellucida is formed and the granulosa cells surrounding the oocyte undergo mitosis and further differentiation. As shown in Figure 2.8, a primary follicle is transformed into a secondary follicle when there are several layers of granulosa cells. Preantral follicles (primary and secondary follicles) become antral (tertiary) follicles, when fluid from the granulosa cells of secondary follicles coalesces to form an antrum (Evans, 2007).

Cyclic increases in FSH concentrations facilitate recruitment of antral follicles. Granulosa cells can produce activin which is thought to provide positive feedback to the anterior pituitary, further increasing gonadotropic FSH secretion (Evans, 2007; Senger, 2007). Recruited antral follicles, which are gonadotropin sensitive, undergo several waves of follicular development beginning in metestrus and ending in proestrus. In subprimates, the final wave of one or more dominant follicles, destined for ovulation, rather than atresia, produces the large amounts of estrogens typical of estrus and required for sexual receptivity and the preovulatory estrous surges in GnRH and LH secretion in subprimates.

Ovarian follicular synthesis of estrogens

The production of estrogens (predominantly estradiol) by antral follicles is accomplished by a mechanism termed the "two-cell or two-gonadotropin model", which can vary somewhat between species (Evans, 2007; Senger, 2007). Cells from the theca interna and/or granulosa cells (depending on the species) produce progesterone from pregnenolone synthesized from cholesterol and, under the influence of relatively low concentrations of LH, theca interna cells convert this

progesterone into androgens and, ultimately, testosterone. In granulosa cells (reportedly theca interna cells in some species), the release of FSH from the anterior pituitary induces aromatase mediated conversion of testosterone produced in the theca cells into estradiol. Stimulation of aromatase activity by xenobiotics can have an overall estrogenic effect on exposed animals (increased production of estradiol).

The effects of estrogenic feedback on the hypothalamic–pituitary–gonadal axis

Increasing concentrations of estrogens associated with estrus alter the hypothalamic GnRH secretory pattern or act on the anterior pituitary itself (Figures 2.3 and 2.8) and decrease pituitary secretion of FSH, while greatly increasing the amount of LH produced and released by the anterior pituitary gland (preovulatory LH surge). Although inhibin produced by granulosa cells further decreases FSH secretion, dominant follicles surviving to estrus do not undergo atresia because of an enhanced sensitivity to basal (FSH) levels. Xenoestrogens have the potential to either imitate or inhibit these estradiol feedback mechanisms in sexually mature females, depending on amount of estrogenic xenobiotic, the endocrine milieu at the time of the exposure and the relative binding affinity of the xenobiotic for estrogen receptors.

Ovulation

The granulosa cells in the one or more dominant follicles (Graafian follicles) cease to divide shortly prior to ovulation and undergo further differentiation, with increased numbers (i.e., upregulation) of LH receptors responsive to the estrogen-induced preovulatory LH surge (Evans *et al.*, 1997; Senger, 2007). As LH increases, granulosa cells (theca interna cells in some species) continue to convert pregnenolone to progesterone, but estradiol production decreases, resulting in a slight preovulatory decline in estradiol. The preovulatory LH surge is associated with increased follicular pressure, degeneration of theca cells and weakening of the follicular wall, completion of the first meiotic division within the oocyte (end of meiotic inhibition except in dogs and foxes) and, finally, ovulation of a secondary oocyte arrested in metaphase II. In felids, ferrets, mink, camelids and rabbits, the preovulatory LH surge is induced by copulation (intromission or vaginal stimulation in most induced ovulators; seminal fluid in camelids). Toxicants which interfere with copulation or sexual contact in these species can interfere with the ovulatory process (Evans, 2007).

Formation and function of a corpus luteum (CL)

Following ovulation, a cascade of endocrine changes takes place in the female subprimate which facilitates the transition from sexual receptivity to non-receptivity. Once an ovulation occurs, blood concentrations of follicular estradiol and inhibin return to their basal levels, and granulosa cells continue their growth, differentiation and increased production and release of progesterone (luteinization) under the influence of LH (Evans *et al.*, 1997; Senger, 2007). The functional ovarian structure which eventually develops from each ovulated follicle is a corpus luteum (often abbreviated CL), which is comprised of large and small luteal cells derived from the granulosa and theca interna cells (granulosa cells in horses), respectively. In most species, luteal cells are responsive to LH and produce progesterone until, shortly before the usual end of diestrus in non-pregnant animals (i.e., late secretory phase in higher primates), the corpus luteum undergoes luteolysis. While the induction of luteolysis is an intraovarian event in higher primates, luteal regression in non-pregnant subprimates is mediated by oxytocin-stimulated production of the luteolysin, prostaglandins $F_{2\alpha}$ ($PGF_{2\alpha}$). Xenobiotics, which can cause endometritis or mimic the actions of oxytocin or $PGF_{2\alpha}$, such as endotoxin or lipopolysaccharide (LPS), can be associated with premature luteolysis. Conversely, toxicants with the opposite oxytocin/$PGF_{2\alpha}$-related effects would be expected to disrupt normal reproductive cyclicity by prolonging the lifespan of the CL and causing a prolonged diestrus or pseudopregnancy (e.g., xenoestrogens in swine) (Evans, 2007).

Species of animals can vary in the number of fertile ovulations and, therefore, corpora lutea which are characteristically associated with each estrous cycle. Monotocous mammalian species usually only ovulate a single secondary oocyte each estrous cycle. The ovaries of litter-bearing (polytocous) mammals generally develop multiple follicles which mature, ovulate and form functional corpora lutea.

Summary of the effects of estrogens and progesterone during the female reproductive cycle

The endocrine changes which occur during the estrous cycle are reflected in behavior and the size, morphology, position and function of the tubular genitalia. As noted in Figures 2.3 and 2.8, estrogens have multiple effects on the female reproductive tract, as well as organ systems, which include: (1) interactions with the hypothalamus and anterior pituitary to alter the patterns of GnRH and gonadotropin secretion which govern follicular development and ovulation; (2) facilitation of sexual receptivity, especially in subprimates; (3) increased blood flow to the reproductive tract; (4) genital swelling; (5) leukocytosis; (6) mucosal secretion and myometrial tone; (7) proliferation and/or keritinization of luminal and/or glandular epithelium within the tubular genitalia; (8) altered electrical conductivity of mucosal secretions; (9) the initiation of the growth of endometrial and mammary glands; and (10) regulation of bone metabolism (Evans, 2007; Senger, 2007). Like estrogens, progesterone also has several effects on the reproductive tract of the female, but the effects of progesterone generally oppose those of estrogens, favoring pregnancy maintenance and sexual non-receptivity, especially in subprimates, over ovulation and appropriately timed sexual receptivity associated with estrogenic stimulation. Progesterone is generally associated with negative feedback to the hypothalamus and anterior pituitary gland which limits GnRH and gonadotropin secretion. Sexual receptivity in subprimates and myometrial contractility and tone are diminished in an endocrine environment dominated by progesterone, while mammary and endometrial gland development and secretion are promoted. Toxicants which disrupt the communication and coordination between the ovary and the other parts of the reproductive tract (e.g., xenoestrogens, xenoandrogens and antiestrogens) will alter the appearance and function of the reproductive organs and can interfere with survival of the oocyte, embryo and/or fetus (Evans, 2007).

Oocyte/sperm transport, normal capacitation of sperm and fertilization

Transport of the ovulated oocyte

The primary reproductive organs involved in the transport of ovulated secondary oocytes (primary oocytes in the bitch) are the oviducts or uterine tubes. Each oviduct consists of an infundibulum, isthmus and ampulla, which have some distinct differences in structure, as well as function (Evans *et al.*, 1997). The ovulated ovum enters the funnel-like opening to infundibulum and is transported to the ampulla or ampullary–isthmic junction for fertilization. Unlike spermatozoa which can generally survive for several days in the oviduct, secondary oocytes usually, depending on the species, are viable for 12 to 24 hours (Evans, 2007; Genuth, 2004b). The appropriate endocrine environment is required for adequate oviductal entry and transport of ovulated oocytes to the site of fertilization. Delayed transport of oocytes within the uterine tubes can result in the death of ova before contact can be made with fertile spermatozoa.

Transport and capacitation of spermatozoa

Transport of spermatozoa

During mammalian copulation, mature sperm stored in the caudae epididymidies travel through the ductus deferens and penile urethra to be ejaculated into the anterior vagina, cervix or uterine body of the female reproductive tract, depending on the species. Spermatozoa can be lost from the female reproductive tract by retrograde loss and phagocytosis by leukocytes (Senger, 2007). Contractions of the smooth muscle within the tubular genitalia (muscularis), as well as interactions involving components of the seminal fluid and luminal secretions of the female reproductive tract, facilitate the transport of sperm to the oviducts (uterine tubes) where, depending on the species, fertilization takes place in the ampulla or at the junction of the ampulla and the isthmus (ampullary–isthmic junction) (Genuth, 2004b; Senger, 2007). While sperm can be rapidly transported to the ampullary–isthmic junction or ampullae of the oviducts (uterine tubes) within minutes of natural or artificial insemination, the relatively slow, sustained transport of motile sperm from reservoirs of spermatozoa in the cervix and uterotubal junctions is the primary mechanism by which the viable sperm that can participate in fertilization actually enter the oviducts (Senger, 2007). Xenobiotics which interfere with the endocrine milieu required for appropriate muscularis contractility and the cervical and uterine mucosal secretions which facilitate sperm transport (e.g., phytoestrogens in sheep) can prevent spermatozoa from getting to the site of fertilization in a timely manner (Evans, 2007).

Capacitation of spermatozoa

Spermatozoa can generally survive in the oviducts (uterine tubes) for several days following insemination. Ejaculated sperm are not competent either to bind to the zona pellucida or to undergo the acrosomal (acrosome) reaction, both of which are required for fertilization of ova by mature spermatozoa. Sperm must be capacitated in order to interact with the ovum. The capacitation process involves calcium influx and biochemical changes to the sperm plasma membrane which result in the "removal" or modification of epididymal and seminal plasma proteins and the exposure of the surface molecules required for spermatozoal binding to the zona pellucida of the ovulated secondary oocyte (Genuth, 2004b; Senger, 2007). Depending on the species and, to some extent, the site of their deposition, spermatozoa become capacitated within the cervix, uterus and/or the oviduct or uterine tube (Senger, 2007).

Fertilization

Fertilization of secondary oocytes by capacitated sperm is a complex process involving a cascade of events which prevents fertilization of an ovum by more than one sperm (polyspermy) and ends in the fusion of the male and female pronuclei (syngamy). In the oviductal ampulla or at the ampullary–isthmic junction, the motility of capacitated sperm becomes hyperactive, facilitating the precise sequence of events which includes the following in their respective order: (1) sperm binding to the zona pellucida of the oocyte involving interactions between species-specific sperm and oocyte proteins; (2) the sperm acrosomal reaction, which results in the release of acrosomal enzymes and exposure of the equatorial segment of the sperm plasma membrane; (3) acrosomal enzyme-associated penetration of zona pellucida by a single spermatozoon; (4) fusion of the plasma membrane of the sperm at its equatorial segment with the plasma membrane of the oocyte; (5) membrane fusion-associated sperm engulfment and the oocyte cortical reaction, which prevents additional oocyte zona binding and membrane fusion (i.e., polyspermy prevention); (6) female pronucleus formation and completion of meiosis; (7) decondensation within the sperm nucleus and male pronucleus formation; and (8) the fusion of male and female pronuclei or syngamy which produces a zygote ready to undergo embryogenesis (Evans, 2007; Genuth, 2004b; Senger, 2007). From the complexity of the fertilization process, it should be apparent that toxicants which result in direct, subtle aberrations in sperm and oocyte formation and maturation can have profound, indirect effects on gamete formation and, potentially, even later downstream changes in embryonic development.

Important aspects of normal embryonic and fetal development

Historical perspective

It should be very evident from Figure 2.2C that, although the depicted newborn has some adult-like qualities, there was a basic understanding of the processes involved in fetal development and nutrition, as well as parturition several hundred years ago. What has really changed over the last century is our understanding of early embryonic development and the signaling pathways which result in the establishment of healthy pregnancies and the delivery of normally developed neonates.

Blastocyst formation and differentiation of the germ cell layers

In order for a zygote to develop into a viable offspring, multiple steps involving cellular division, migration, differentiation and organization must take place. Embryonic

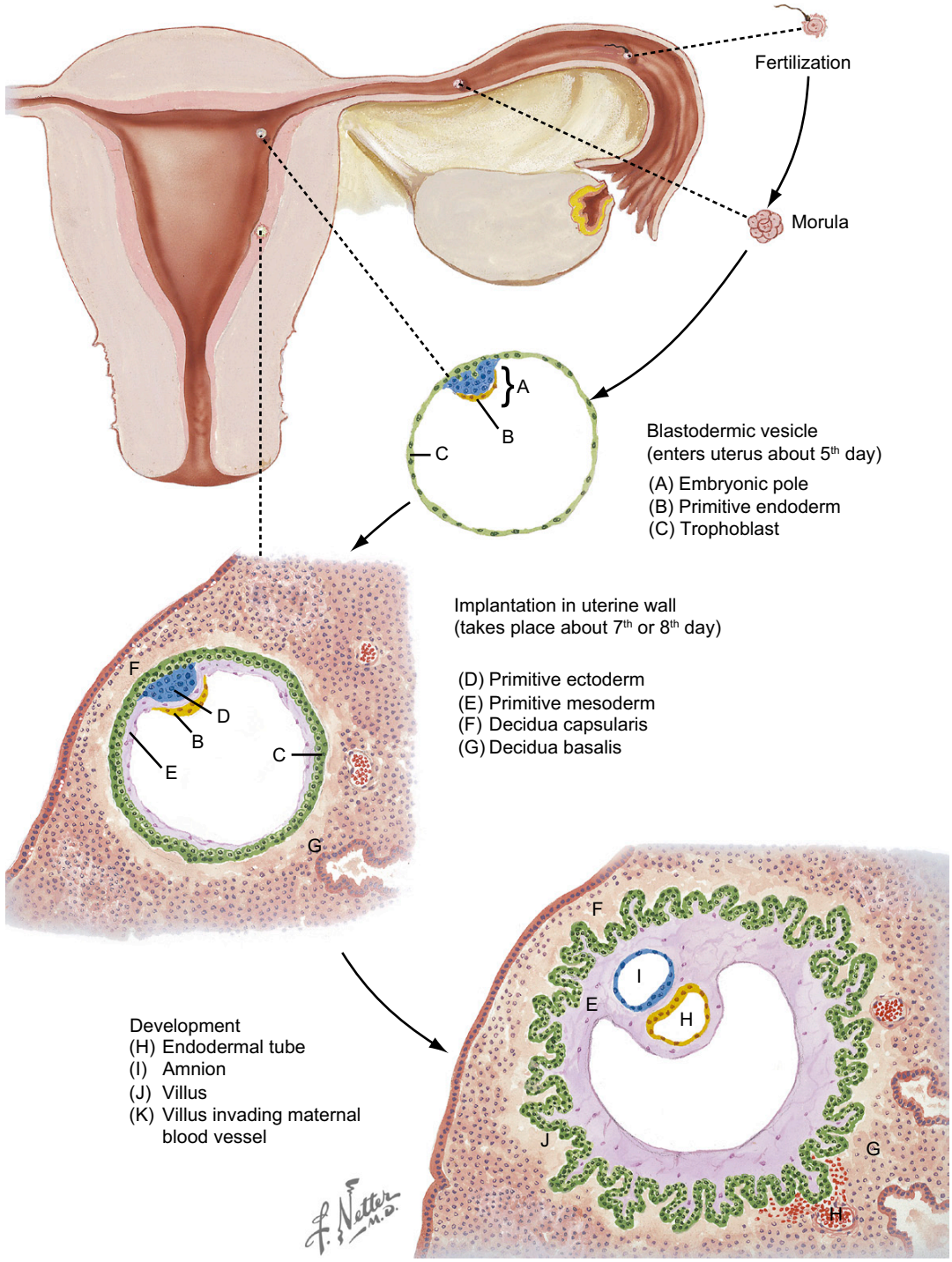

FIGURE 2.9 The series of developmental events associated with embryogenesis in humans, which occur after fertilization, including implantation and initial formation of the amnion and chorionic villi, are shown. Figure was obtained and modified, with permission, from Netter (1997).

and fetal survival requires that these various steps take place in a precise order and at set times during the gestation of each species. Within 24 hours following fertilization, the zygote located in the oviduct begins to divide, within the confines of the zona pellucida, into multiple blastomeres, which ultimately form a ball of cells referred to as the morula (Evans, 2007; Senger, 2007). As shown in Figure 2.9, a fluid-filled cavity (blastocoele) develops within the developing embryo, and the newly formed blastocyst, which is divided into cells forming either the inner cell mass (future embryo proper) or the trophoblast (future chorion), enters the uterus. In humans, the entry of the blastocyst or blastodermic vesicle into the uterus generally occurs on day 5 after ovulation and "hatches" from the zona pellucida on approximately day 6 (Evans, 2007; Genuth, 2004b; Netter, 1997).

"Maternal recognition of pregnancy" and implantation

The conceptus and, in most cases, the trophoblastic cells of most mammalian embryos, other than those for which the timing of luteolysis and duration of pregnancy are very similar to one another (i.e., dogs and cats), must produce some signal to prevent luteolysis (i.e., entry into the next estrus or, in the case of higher primates, menses) and to maintain luteal phase progesterone concentrations until an alternative source of progestagens develops (Evans, 2007; Senger, 2007). In subprimates, this process, which is also referred to as "maternal recognition of pregnancy", involves species-specific embryo–endometrium interactions which prevent the production or redirect the release of endometrial $PGF_{2\alpha}$. Embryonic production of species-specific interferon-τ, o-IFN-τ- and b-IFN-τ, prevents luteolysis in sheep and cattle, respectively, by inhibiting the synthesis of $PGF_{2\alpha}$. In swine, estrogen secretion by porcine embryos appears to prevent luteolysis by redirecting the release of $PGF_{2\alpha}$ away from the ovarian circulation. Embryonic intrauterine migration appears to prevent luteolysis and maintain luteal production of progesterone in equids.

Higher primates, such as humans, present a different set of circumstances, with respect to "maternal recognition of pregnancy" and maintenance of the corpus luteum. In these species, the endometrium does not appear to have an essential role in luteolysis, and the regulation of luteal regression appears to be an intraovarian event. Therefore, the blastocysts of higher primates must produce some "signal", which directly interacts with the maternal ovaries, in order to facilitate "maternal recognition of pregnancy" and prevent luteolysis. It is interesting to note that the embryos of these species of "higher" mammals undergo true implantation within the maternal endometrium, rather than the "attachment" which is observed in large domestic mammals, and that specialized cells involved in implantation play a pivotal role in preventing luteolysis.

Shortly after entry into the uterus during the secretory phase of the menstrual cycle, the human blastocyst attaches to the pregravid endometrium (i.e., the early decidua or the hormonally stimulated lining of the endometrium which will eventually form the maternal component of the placenta) (Netter, 1997). Very soon thereafter, in the mid- to late-secretory phase (i.e., luteal phase), fibroblast-type, stromal cells, located near uterine blood vessels, increase in size and accumulate glycogen and lipid to form decidual cells, which are only maintained if pregnancy occurs. By approximately day 7 or 8 after ovulation, the blastocyst has penetrated the luminal epithelium of the uterus, and the invasive capabilities of the trophoblastic cells of the blastocyst, specifically the syncytiotrophoblasts, have enabled the implantation of the blastocyst within the endometrium, surrounded by decidual cells (Figure 2.9) (Foster and Gray, 2008; Netter, 1997). On day 9 after ovulation, the syncytiotrophoblastic cells begin to secrete human chorionic gonadotropin (hCG) which, because of its LH-like activity, "rescues" the corpus luteum from luteolysis and increases the luteal production of progesterone, as well as estrogens. The increased secretion of these hormones, especially progesterone, stimulates widespread "decidualization" of the uterine stroma and atrophy of endometrial glands (Foster and Gray, 2008; Genuth, 2004b; Netter, 1997). Concurrent with these events, the syncytiotrophoblasts continue their invasion of the endometrium and, in particular, the uterine vasculature, to provide for the future nourishment and growth of the developing embryo and fetus. In other species of animals (e.g., rodents) where there is also actual implantation versus simple "attachment" of the embryo, the same, basic sequence of events (i.e., attachment to the endometrium, epithelial penetration, decidualization, and trophoblastic invasion into the uterine vasculature) also take place, including the production of chorionic gonadotropin and placental lactogen.

Formation of the extraembryonic membranes

Concepts and definitions

Most mammalian species are "eutherian", and during pregnancy form a placenta which is comprised of both maternal and fetal components. The term "decidua" is generally used in reference to humans and higher primates and can be used to refer to the lining of the endometrium which is shed during menses. However, "decidua" is used more frequently in connection with the maternal portion of the placenta which is shed at birth. The portion of the decidua which interdigitates with the trophoblast and, eventually, the chorion, is referred to as the decidua basalis. This portion of the deciduas provides nourishment to the embryo until formal connections to maternal vascular channels are established and a single, central circulation is formed (Genuth, 2004b). The portion of the decidua which surrounds the human embryo and, later, the fetus is the decidua capsularis. The decidua vera or decidua parietalis refers to the rest of the endometrial lining which is shed at birth but which does not interact with the trophoblatic cells or chorion (Figure 2.9) (Netter, 1997).

The yolk sac, amnion, allantois and chorion are the extraembryonic membranes formed by the mammalian embryo (Senger, 2007). While the yolk sac in most mammalian species normally undergoes regression (early in pregnancy in higher primates; later in rodents and rabbits), the allantois and chorion generally fuse to form the allantochorion, and the fluid-filled amnion provides a shock-absorbing, aquatic environment to facilitate fetal development and transport (Evans, 2007; Foster and Gray, 2008; Senger, 2007). The allantochorionic membrane is the fetal contribution to the placenta and the chorionic villi are the structures which interdigitate with various layers of the maternal endometrium which are maintained during pregnancy (Evans, 2007; Foster and Gray, 2008; Senger, 2007). In higher primates, the placental circulation and hemotrophic nutrition are established very early.

Placental types

Mammalian placentation can be classified according to the degree of intimacy between the maternal and fetal circulations (i.e., the number of tissue layers separating maternal and fetal blood) and by the pattern of distribution of the chorionic villi on the surface of the placenta facing the maternal endometrium. Epitheliochorial placentae have a total of six layers separating the maternal and fetal circulations and are observed in a variety of species, including equids and swine. Ruminant placentation is described as syndesmochorial because of the transient erosion and regrowth of the maternal epithelium, which results in the intermittent exposure of maternal endothelium (capillaries) to chorionic epithelium (Foster and Gray, 2008; Senger, 2007). Canine and feline