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CHAPTER 22

The Epididymis

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ABSTRACT

Throughout embryonic and early postnatal development, the mammalian epididymis changes from a straight tube to a highly coiled, complex duct that links the efferent ducts to the vas deferens. Overwhelming evidence points to the importance of this tissue in transforming spermatozoa leaving the testis as immotile cells, unable to fertilize oocytes, into fully mature cells that have the ability both to swim and to recognize and fertilize eggs. Under normal conditions, the acquisition of these functions is essentially completed by the time sperm enter the proximal cauda epididymidis. In addition to sperm maturation, the epididymis also plays an important role in sperm transport, concentration, protection, and storage. A highly specialized and region-specific microenvironment is created along the epididymal lumen by active secretion and absorption of water, ions, organic solutes, and proteins as well as by the blood–epididymis barrier. The primary factor regulating epididymal function is androgens, but there is mounting evidence that estrogens, retinoids, and other factors coming directly into the epididymis from the testis through the efferent ducts, such as growth factors, also play specific regulatory roles. Several epithelial cell types,

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each showing selective expression of genes and proteins, are differentially distributed along the duct; each cell type shows highly regionalized expression of a wide array of markers. Both epididymal epithelial cells and spermatozoa in the lumen are targets for xenobiotics; such exposures can result in undesirable toxic effects or may provide the basis for the development of novel male contraceptive agents. During aging, both the epididymal epithelium and the germ cells in the lumen undergo a series of dramatic changes. The explosion of knowledge we are witnessing regarding all aspects of epididymal structure and function is likely to lay the basis for a new fundamental understanding of epididymal cell biology and novel therapeutic approaches targeted at this organ.

INTRODUCTION

Since the publication of the comprehensive review on the male excurrent duct system (efferent ducts, epididymis, and vas deferens) in the first edition of *Physiology of Reproduction* in 1988 (1), a remarkable series of events has marked the growth in our knowledge and understanding of this duct system. At that time, the epididymis, a long, complex, convoluted duct connecting the efferent ducts to the vas deferens, was viewed as having moved from being "an abandoned child" of the male reproductive system (2), to a point of maturity, where its basic structures, functions, and regulation were beginning to be understood.

Since 1988, three international conferences dedicated to this tissue have been held, with proceedings ensuing for two of these (3,4), and the first comprehensive, multiauthored volume dedicated to the epididymis has been published (5). The yearly number of research publications on this tissue has increased by more than one order of magnitude (from less than 500 to more than 6,000) since the review appeared in the first edition of *Physiology of Reproduction*. Therefore, in preparing the current review, we have chosen first to narrow the scope by placing most of the emphasis on the epididymis itself, as opposed to the other components of the excurrent duct system; second, to exclude some topics that have been extensively reviewed recently, such as changes in spermatozoa during epididymal transit, innervation and vasculature of the duct system, or pathology of the epididymis; and third, to focus on some of the more exciting recent developments in this field and provide supplemental information in an alternative format. Several of the subjects covered in the first edition are updated, but, because of space limitations, we have chosen not to include most of the plates that depict the histological organization and structure of the various epididymal epithelial cells from the first edition, but rather to place them on a web site (www.medicine.mcgill.ca/PhysiolReprodThirdEd/Epididymis). Along with these plates, this web site contains color versions of plates from the current chapter and a number of tables, including some containing detailed information about specific epididymal proteins and genes as well as their regulation. We have intentionally chosen not to include such tables in the chapter not only because of space limitations but because of the need to update this information regularly. We also felt that the review should focus on our ideas, philosophy, and biased judgment on what we believe is important and where the field should go.

HISTORICAL PERSPECTIVE

As early as the 4th century B.C., the epididymis was described by Aristotle in his *Historia Animalium*, whereas the first recorded description of a dissected epididymis was made by de Graaf in 1668 in his monograph *Tractatus de Virorum Organis Generationi Inservientibus* [reviewed in Orgebin-Crist (6)]. De Graaf noted that "the semen … was watery and ash-like in the testis and becomes milky and thick in the epididymis" (7). Between 1888 and 1928, a number of scientists described the histological features of the epididymal epithelium, and surmised, from the images of secretion they observed, that epididymal secretions "nourished" the spermatozoa in the epididymal lumen. In 1913, Tournade (8) showed that spermatozoa released from the proximal epididymis were not motile when diluted in saline, but spermatozoa released from the distal epididymis were fully motile. In the bat, spermatozoa were observed to survive for several months in the cauda epididymidis during hibernation, presumably protected by epididymal secretions (9), whereas rabbit spermatozoa survived in the epididymis for 30 to 60 days (10), and bull spermatozoa for 2 months (11), after ligation of the efferent ducts. The implication of these observations was that the epididymis conditioned the development of sperm motility and was important for sperm survival.

The consensus of these early investigations can be summarized by the last sentence of Benoit's classic 1926 (12) monograph: "The role of epididymal secretions is to maintain sperm vitality, to permit the development of sperm motility, and possibly to protect them against noxious agents." In a series of four papers between 1929 and 1931, Young (13–16) showed that during epididymal transit spermatozoa not only acquire a mature motility pattern but become fertile; based on some poorly designed and interpreted studies, he concluded, erroneously, that the changes spermatozoa undergo during their transit through the

epididymis represent a continuation of changes that start while spermatozoa are still attached to the germinal epithelium, and are not conditioned by some specific action of the epididymal secretion (6). Nevertheless, by 1931 most of the problems and questions relating to epididymal physiology had been recognized, and most of the work done since then has been to provide the experimental evidence to flesh out the insights of earlier investigators.

Few studies were published on epididymal physiology until the 1960s. Those that appeared focused primarily on establishing the length of time necessary for spermatozoa to transit through the epididymis (17,18). The apparent lack of interest in the epididymis during this 20- to 30-year span is puzzling. After the work of Benoit in 1926 (12) and Young in 1931 (13–16), there was a clear controversy that needed to be resolved. In 1965, only 43 papers on the epididymis were published. Whatever the reason for this disaffection, in 1964 Thaddeus Mann, in his book *The Biochemistry of Semen* (2), refers to the epididymis as the "abandoned child" of the reproductive system.

In the mid- and late 1960s, a resurgence of interest in the epididymis was spearheaded independently by Orgebin-Crist and Bedford; they demonstrated that the key event in sperm maturation was not the passage of time, as proposed by Young, but exposure to the luminal environment of the epididymis (19,20). Thanks to these and other studies, by the end of the 1960s it was established that the potential for sperm motility and fertilizing ability is acquired as spermatozoa pass from the proximal to the distal epididymis; that the maturation process does not end with the acquisition of fertilizing ability, because spermatozoa that have just become fertile induce a higher rate of embryonic mortality when inseminated in vivo; that the maturation process depends on an androgen-stimulated epididymis; and that the maturation process includes changes in sperm organelles [reviewed in Orgebin-Crist (21)].

Since the 1980s, we have seen an explosion of studies on the presence, characterization, immunolocalization, and regulation of a large number of proteins and their RNAs known to be specific to the epididymis, to be expressed at particularly high levels in some segments of the tissue, or to control key molecules that regulate epididymal function. The objective of many of these studies has been to develop ways of modifying epididymal function with respect to rendering spermatozoa fully mature and motile, thus providing potential leads for male contraception or the management of male infertility. In the course of these studies, it has become clear that this tissue presents a novel model for studying cell-, segment-, and region-specific gene expression, for understanding mechanisms of aging, and for identifying processes conferring selective protection from infections and cancer.

DEVELOPMENT OF THE EPIDIDYMIS

Formation of the Mesonephric/Wolffian/Nephric Duct and Tubules

Many of the studies focusing on the specification and regulation of mesonephric duct and tubule formation have been conducted on chick, frog, and zebrafish embryos, with some more recent studies using the mouse embryo. Space limitations prevent a comprehensive review of the development of the urogenital system, but there are several excellent resources with detailed descriptions of these events (22,23). What follows is a basic summary of the embryonic and postnatal development of the epididymis.

The urogenital system is derived from the intermediate mesoderm, which, in the chick and mouse, is the result of a complex interaction between the intermediate and paraxial mesoderm. The expression of two key transcription factors, Pax 2 and Pax 8, plays a vital role in this process because mice that lack these two genes fail to form a mesonephros and, therefore, later structures of the urogenital system; Bouchard et al. (2002) (24) suggest that Pax2 and Pax8 are critical regulators that specify the nephric lineage. At approximately gestational days 8 to 9 in the mouse and stage 16 in the developing chick, the mesonephric (Wolffian/nephric) duct develops as a cord of epithelial cells that express c-Sim-1 (chick) and Pax2 (25,26), undergoes the formation of a lumen, and rapidly extends throughout the length of the embryo in a cranial-to-caudal direction. There is considerable evidence to suggest that, in some species, elongation of the nephric duct is the result of cell rearrangements rather than proliferation or changes in cell shape (27). Interestingly, for proper nephric duct formation but not elongation, bone morphogenetic protein-4 expression in the surface ectoderm appears to be critical (28). Retinoic acid is also crucial for nephric duct formation because the duct does not form in RALDH2, a retinoic acid synthetic enzyme, knockout mice (29). As the mesonephric duct elongates, it induces the nearby mesenchyme to form the mesonephric tubules (30). The tubules have a characteristic J- or S-shape and resemble developing nephrons. Without the Wolffian duct, the mesonephric tubules do not form (31).

It is clear that those cranial mesonephric tubules that lie close to the testis survive, and the distal end grows toward the gonad, whereas the proximal end

contacts the wolffian/mesonephric duct. However, several fundamental questions remain. What are mechanisms by which the mesonephric tubules are induced by the mesonephric duct? This process involves a complex mesenchyme-to-epithelial transition, and there is evidence to suggest that leukemia inhibitory factor, members of the Wnt family (Wnt6 and Wnt4), and fibroblast growth factor (FGF)-2 are responsible for mesenchymal cell aggregation and the formation of the renal nephron [reviewed in Gilbert (22)]. Whether this is recapitulated during the formation of the mesonephric tubules is unclear. Alarid et al. (1991) (32) have shown that FGF-2 is important for epididymal development because the epididymis fails to develop when embryonic urogenital ridges and sinuses are cultured under the kidney capsule in the presence of anti–FGF-2 antibodies. Studies by Sainio et al. (1997) (33) showed that the Wilms tumor-1 (*WT1*) gene is important in the formation of the most caudal mesonephric tubules.

What are the mechanisms by which only those tubules close to the gonad survive, yet others cranial and caudal to the gonad degenerate? Evidence suggests that the caudal tubules normally undergo apoptosis, so presumably those tubules close to the gonad express the antiapoptotic genes required to survive, but this has not been established.

What controls the migration and differentiation of cells of the distal tubule into the rete testis? Earlier studies by Upadhyay et al. (34) provided evidence that in the mouse, the cells in the distal end of the mesonephric tubules contribute directly to the rete testis and undergo differentiation, and that fusion between the mesonephric tubules and the testis did not occur. This finding is perhaps not too surprising in light of the extensive contribution of the mesonephros to the developing testis.

Which portions of the mesonephric tubule and duct form the discrete regions of the initial segment and the first part of the caput epididymidis? There is some evidence to suggest that cells of the wolffian duct contribute to a small part of the proximal portion of the mesonephric tubule but, again, it is not clear whether this represents the junction between the caput and the initial segment. For a more complete overview of these processes, the reader should refer to the reviews by Sainio (2003) (31), Vazquez et al. (2003) (35), and Jones (2003) (36).

It is intriguing that the development of the mesonephric tubules may recapitulate the development of the renal vesicle into the renal proximal and distal tubules. It would not be too surprising to find that many genes that regulate this process also play a role in the formation of the efferent ducts or initial segment. For example, members of the Wnt family [Wnt 4 (37)] and cadherins (38) play a role in the proximal/distal patterning of the renal proximal and distal tubules. Cadherin 6 is expressed in the proximal tubule progenitors, E-cadherin is expressed in the distal tubule, and P-cadherin is expressed in the glomerulus (39). The initial segment fails to develop in c-Ros mutants (40) and in Sxr (XXSxr) mice (41), suggesting that the defect may lie in the origin or development of the mesonephric tubules. Although it is well recognized that androgens play an important role in epididymal development, the initial segment fails to develop in a normal androgen environment in the Sxr mouse (41). This would suggest that additional factors, such as growth factors, may be important for mesonephric tubule/initial segment development. In both of these mutants the prominent vascular system that supplies the initial segment also fails to develop (40,42), indicating that there is an intimate relationship between epithelial and endothelial development. Again, this is perhaps not surprising in view of the formation of the renal glomerulus at the tip of the renal proximal tubule where endothelial cells are recruited to this site.

The embryonic origins of the different regions of the epididymis have received some interest; it appears that the efferent ducts are derived from the mesonephric tubules, whereas the caput epididymidis to the vas deferens regions are derived from the mesonephric duct [reviewed in Jones (36)]. However, the origins of the initial segment warrant further investigation because it is not entirely clear if the cells are of mesonephric tubule or duct origin. The cells of the initial segment are quite distinct from either the efferent ducts or the distal epididymal regions, but function similarly to the efferent ducts in that they are actively involved in water reabsorption. Hence, it is possible that the initial segment may be of mesonephric tubule origin.

Postnatal Development

A more complete picture of the postnatal development of the epididymis has emerged over the years, with the majority of studies focused on the mouse, rat, and human, although other species have been studied, including the bull, dog, rabbit, marmoset, and boar [reviewed in Rodriguez et al. (43)]. Perhaps the most detailed description of postnatal epididymal development is that of the rat, and the studies by Sun and Flickinger (1979) (44) and Hermo et al. (1992) (45) provide the basis for the following brief overview. There appear to be three major stages of epididymal development: the undifferentiated period, the period of differentiation, and the period of expansion.

By birth, the epididymis has undergone considerable coiling in the proximal regions (initial segment to corpus) and the cauda has yet to complete coiling (Fig. 1). In the next 1 to 2 days, individual

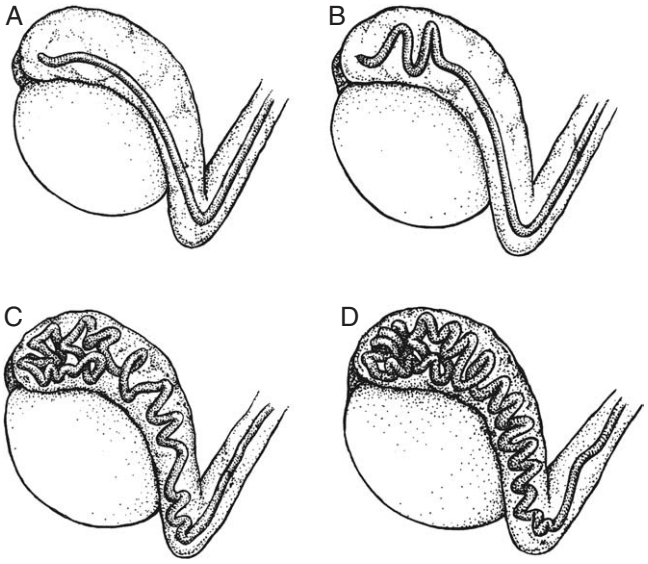

FIG. 1. Coiling of the epididymal duct from mouse E14 to P1. Note that coiling proceeds in a caput-to-cauda direction and is completed in the early postnatal period. The efferent duct–initial segment coiling is not shown here, but proceeds independently of the coiling of the main epididymal duct. To help visualize the changes, the size of the testes and epididymides at different ages are not drawn to scale. (From D. Bomgardner and B. T. Hinton, unpublished observations.)

septa are seen delineating the tubule into segments (D. Bomgardner and B. T. Hinton, unpublished observations). The epithelium is undifferentiated and is characterized by columnar cells containing numerous mitotic figures. There is considerable growth of the rat epididymal duct from its embryological origins to postnatal day 15, when it reaches almost 2 m in length (45,46). The period of differentiation has been examined extensively. Undifferentiated cells differentiate into the classically described cells of the adult epididymis: principal, halo, narrow (pencil), basal, and clear (light) cells; these cells are described later. Key changes in development are following: at postnatal day 14 the halo cells appear, on day 15 one observes narrow and columnar cells, by day 28 the columnar cells differentiate into basal and principal cells, from day 36 onward the narrow and clear cells appear, and by approximately day 49 all epididymal cells are fully differentiated. Figure 2 outlines the differentiation of the rat epididymal epithelium from birth to adulthood. The period of expansion describes the continued growth of the duct and the appearance of spermatozoa in the lumen.

The mechanisms that regulate the growth and differentiation of the epididymal duct are unknown, although it is clear that the expression of many epididymal genes is developmentally regulated. Certainly, luminal and circulating androgens play a critical role, but luminal fluid factors other than androgens also may be important. Early studies by Alexander (1972) (47) and Abe et al. (1984) (48) provided evidence that luminal contents produced by the testis or proximal regions of the epididymal duct may regulate epididymal epithelial differentiation, although the candidate regulators were not identified. Because there is some evidence to suggest that testicular luminal fluid growth

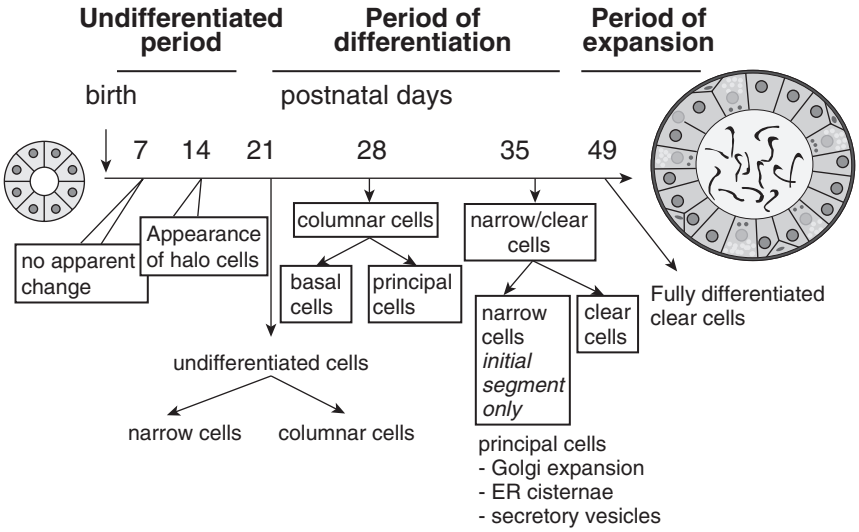

FIG. 2. Diagram outlining the differentiation of the rat epididymal epithelium from birth until adulthood. Epididymal epithelial cells are undifferentiated until approximately day 21, when both narrow and columnar cells are first observed. At approximately day 28, the columnar cells differentiate into principal cells and basal cells. Narrow cells are seen in the initial segment only, but clear cells are observed throughout the epithelium from approximately day 36 onward. By day 49, all epididymal epithelial cells are fully differentiated. (Adapted from Rodriguez, C. M., Kirby, J. L., and Hinton, B. T. [2002]. The development of the epididymis. In *The Epididymis: From Molecules to Clinical Practice* [B. Robaire and B. T. Hinton, Eds.], pp. 251–267. Kluwer Academic/Plenum, New York.)

factors (e.g., FGFs) may regulate initial segment function and that the same growth factors regulate embryonic wolffian duct development, it is tempting to speculate that luminal growth factors (e.g., bone morphogenetic proteins, FGFs, nerve growth factors) may also regulate epididymal epithelial differentiation. One might also envisage a more complex cell–cell interaction in which multiple luminal factors are secreted in the proximal regions of the duct (and testis), which in turn cause the cells in the mid-distal regions (e.g., corpus) to secrete factors, which in turn regulate cell function in the more distal epididymal region (e.g., cauda). Such multiple cascades may act to coordinate proper differentiation along the duct.

STRUCTURAL ORGANIZATION OF THE EPIDIDYMIS

Anatomy

Seminiferous tubules converge to form the rete testis, which in turn gives rise to the efferent ducts (ductuli efferentes), a series of 4 to 20 tubules, the number depending on the species (49,50). These tubules converge to form a single highly coiled duct, the epididymis (from the Greek meaning "on or adjacent to the testis"), which is extremely long and varies in length from 1 m in mice, (51), 3 m in rats, (52), 3 to 6 m in humans, (53), and up to 80 m in horses, (54).

The epididymis is usually divided into four gross anatomical regions: the initial segment, head (caput), body (corpus), and tail (cauda), as first described by Benoit (1926) (12). Subsequently, a number of other schemes have been proposed for dividing the epididymis into different regions or segments, including a number of zones and the demonstration of the presence of an intermediate zone between the initial segment and caput epididymidis that has characteristic cells (55–59). However, in this review, we retain the most commonly used nomenclature for the four regions described previously. In all mammalian species examined to date, each region of the epididymis is further organized into lobules separated by connective tissue septa. These septa not only as serve internal support for the organ but have been proposed to provide a functional separation between lobules that allows selective expression of genes and proteins within individual lobules (60). The extension of the epididymis is a straight tube, the vas deferens, which is surrounded by a very thick muscular layer. The vas deferens connects with the urethra, which empties to the outside of the body. A schematic representation of the testis, efferent ducts, epididymis, and vas deferens is shown in Fig. 3.

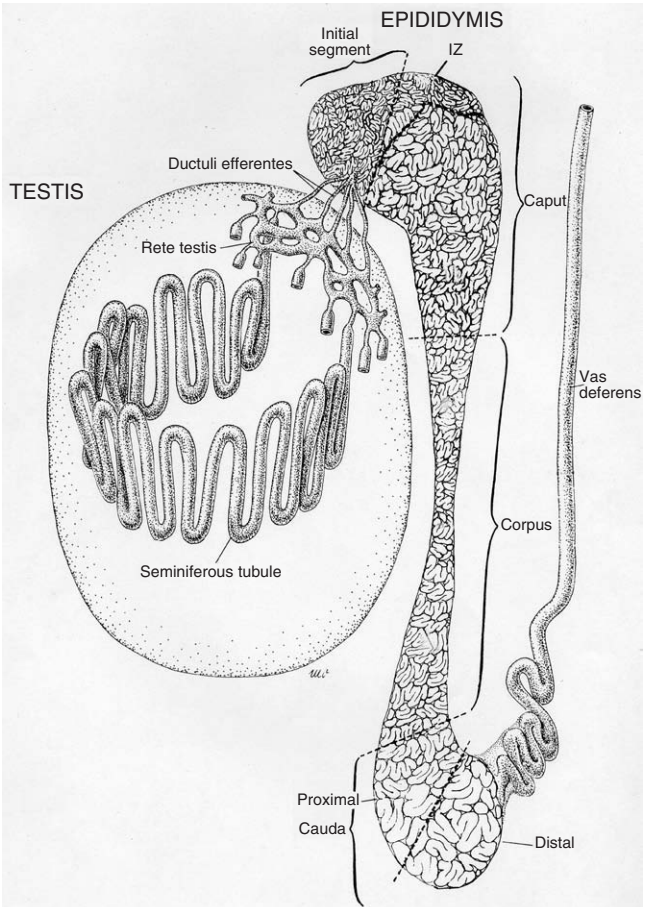

FIG. 3. Diagrammatic representation of the testis showing a seminiferous tubule and the rete testis, the ductuli efferentes, the epididymis, and vas deferens. The major regions of the epididymis (i.e., the initial segment, intermediate zone, caput, corpus, and proximal and distal cauda) are indicated. (Adapted from Robaire, B., and Hermo, L. [1988]. Efferent ducts, epididymis and vas deferens: structure, functions and their regulation. In *The Physiology of Reproduction* [E. Knobil and J. D. Neill, Eds.], pp. 999–1080. Raven Press, New York.)

Epididymal Cell Types and Specific Markers

There are several types of epithelial cells that line the epididymis; some are located throughout the duct (e.g., principal cells), whereas others are found either exclusively or primarily in specific regions (e.g., narrow cells). We provide a brief description of the major cell types along the epididymis, discuss the potential functions of these cells, and demonstrate the presence of specific markers for each cell type. Several detailed reviews of the histology of the epididymis for a number of species ranging from mouse to human, and including dog, camel, elephant, opossum, bull, ram, hamster, mouse, and monkey, have been published (1,12,48,56,57,61–73). Together, these publications provide increasing evidence that similar regions and cell types are present in most mammals, including humans.

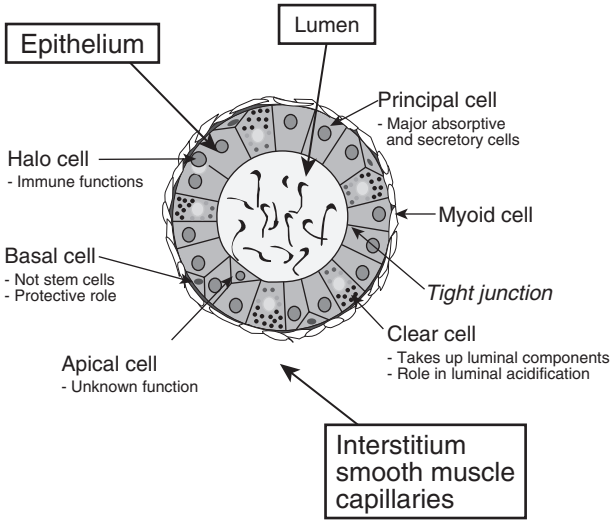

FIG. 4. Schematic organization of the major cell types in the epididymis as observed at the light microscope. The three epididymal compartments as well as the relative position and distribution of each of the main cell types are illustrated. The major functions associated with each cell type are also identified.

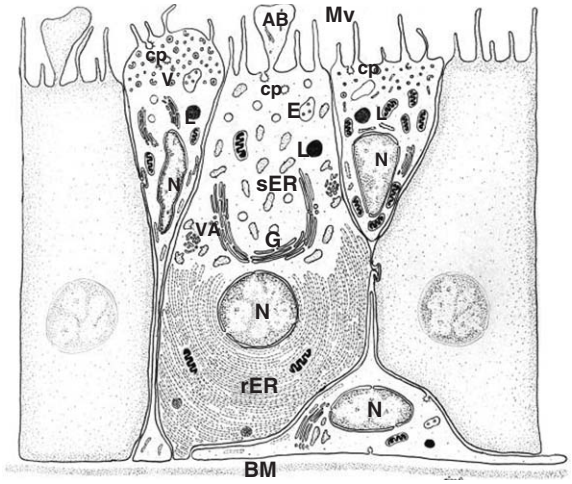

FIG. 5. Schematic representation of cells found in the epithelium of the initial segment of the epididymis, as visualized by the electron microscope. Narrow cells, represented on the left, are elongated cells tapering toward the basement membrane (BM); they show numerous, small, apical cup-shaped vesicles (v), coated pits (cp), and occasional endosomes (E) and lysosomes (L). Principal cells are columnar in appearance and show coated pits (cp), endosomes (E), and lysosomes (L), components of the endocytic apparatus. They also contain numerous parallel cisternae of rough endoplasmic reticulum (rER) located basally and dilated, irregularly shaped cisternae of ER sporadically dispersed in the apical and supranuclear regions of the cell (sER). The Golgi apparatus (G) is elaborate and located supranuclearly. Principal cells show blebs of cytoplasm emanating from their apical cell surface, referred to as apical blebs (AB), and small vesicular tubular aggregates (VA) occupying regions adjacent to the Golgi apparatus. Also illustrated on the left is an apical cell that, unlike the narrow cell, does not extend to the basement membrane and shows few coated pits (cp), apical vesicles, or endosomes and lysosomes (L). A basal cell stretches along the basement membrane and sends a thin process up toward the lumen. N, nucleus; Mv, microvilli. (Reproduced with permission from Hermo, L., and Robaire, B. [2002]. Epididymis cell types and their function. In *The Epididymis: From Molecules to Clinical Practice* [B. Robaire and B. T. Hinton, Eds.], pp. 81–102. Kluwer Academic/Plenum, New York.)

These cell types appear in the appropriate regions, share similar structural features and functions, including regional differences, and show very similar patterns of expression of some secretory proteins (74–79). The comprehensive set of light and electron microscopy plates prepared for this volume are available at www.medicine.mcgill.ca/PhysiolReprodThirdEd/-Epididymis. Schematic representations of the organization of all the cell types and their known functions are shown in Fig. 4. Key components of epithelial cells in the initial segment and in the remainder of the epididymis as observed at the electron microscope are depicted in Figs. 5 and 6, respectively.

Principal Cells

The main cell type in the epididymis of all mammals is referred to as the *principal cell*. These cells appear along the entire duct but show structural differences in each region (1,63). The most striking feature of these cells is their highly developed secretory and endocytic machinery and their basally aligned nuclei. Depending on the segment examined, principal cells comprise approximately 65% to 80% of the total epithelial cell population of the epididymis (80). Both their structure and functions vary dramatically between the different segments (1,63,81). These differences are reflected in the appearance and organization of their secretory apparatus (endoplasmic reticulum, Golgi apparatus, and secretory granules) and endocytic apparatus (coated pits, endosomes, multivesicular bodies, and lysosomes). The precise characterization of the secretory granules and Golgi apparatus of these cells, as well as the identification of organelles and mechanism of uptake of luminal substances, have been well described (82). One other major difference is the abundance of lipid droplets found only in the corpus epididymidis (Fig. 6), the exact significance of which is still poorly understood.

Principal cells synthesize a large number of proteins that are then either retained in the cells or actively secreted into the luminal compartment (1,81,83–89) [reviewed in Robaire et al. (90), Cornwall et al. (92), and Kirchhoff (93)]. They also play an active role in endocytosing proteins found in the luminal compartment of the epididymis [reviewed in Hermo and

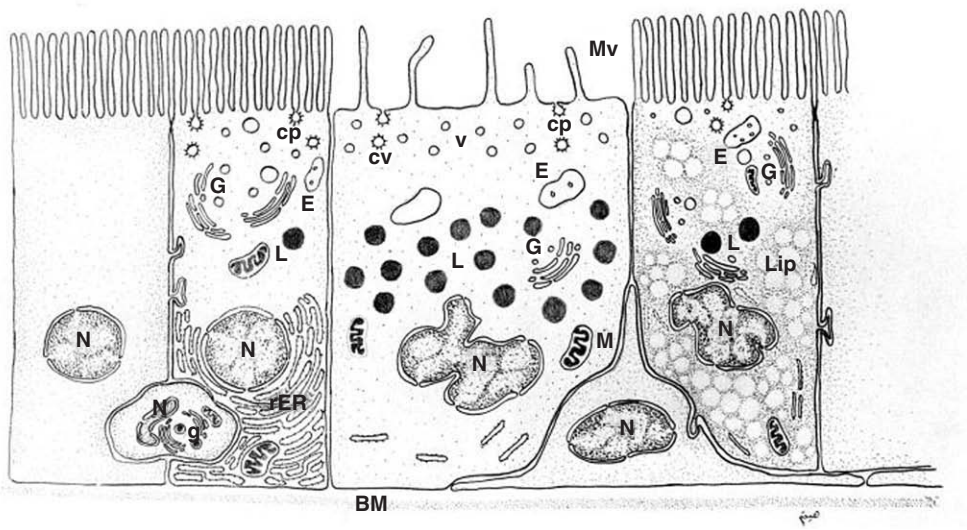

FIG. 6. Schematic representation of a principal cell of the caput epididymidis on the left and a principal cell of the corpus epididymidis on the right, with a clear cell in between, as visualized by the electron microscope. Also represented is a halo cell and a basal cell. Principal cells of both regions contain coated pits (cp), endosomes (E) and lysosomes (L), and an elaborate Golgi apparatus (G). Rough endoplasmic reticulum (rER) occupies the basal region of the principal cell of the caput, whereas numerous lipid droplets (lip) occupy the cytoplasm of the principal cells of the corpus region. The clear cell shows few microvilli (Mv), but numerous coated pits (cp), small apical vesicles (v), endosomes (E), and lysosomes (L), all involved in endocytosis. The halo cell is inserted between adjacent principal cells, is located basally, and contains small dense core granules (g), whereas the basal cell stretches itself along the basement membrane (BM). N, nucleus. (Reproduced with permission from Hermo, L., and Robaire, B. [2002]. Epididymis cell types and their function. In *The Epididymis: From Molecules to Clinical Practice* [B. Robaire and B. T. Hinton, Eds.], pp. 81–102. Kluwer Academic/Plenum, New York.)

Robaire (2002) (82)]. A schematic representation of these processes is depicted in Fig. 7.

Apical Cells

Apical cells are found primarily in the epithelium of the initial segment and intermediate zone (94,95), although they have been seen occasionally in other segments in aging rats (96). These cells have a characteristic apically located spherical nucleus and do not contact the basement membrane (Fig. 5). They differ clearly from adjacent narrow and principal cells in terms of their protein expression profile (95). However, little is known about the specific functions of these cells, aside from their ability to endocytose substances from the lumen, as revealed by the examination of β-hexosaminidase A knockout mice (69), and the observation that they contain many proteolytic enzymes (95).

Narrow Cells

In the rat and mouse, narrow (pencil) cells of the adult epididymis appear only within the epithelium of the initial segment and intermediate zone (94,95). These cells are narrower than the adjacent principal cells, attenuated, and send a thin process of cytoplasm to reach the basement membrane (Fig. 5). They are characterized by numerous apically located cup-shaped vesicles that are involved in endocytosis and function in secreting H^+ ions into the lumen by recycling to and from the apical plasma membrane (97). Similar cells have also been reported in the same regions in numerous other species, including bovine, hamster, echidna, and human (61,65,66,76,95,98). Narrow cells are distinct from apical cells in their morphological appearance, relative distribution, and expression of different proteins. They also differ dramatically from neighboring principal cells and display region-specific expression of proteins such as the glutathione S-transferases and lysosomal enzymes (95).

Clear Cells

Clear cells are large, active endocytic cells present only in the caput, corpus, and cauda regions of the epididymis and are found in many species, including humans (1,63,99). These cells are characterized by an apical region containing numerous coated pits, vesicles, endosomes, multivesicular bodies, and lysosomes and a basal region containing the nucleus and

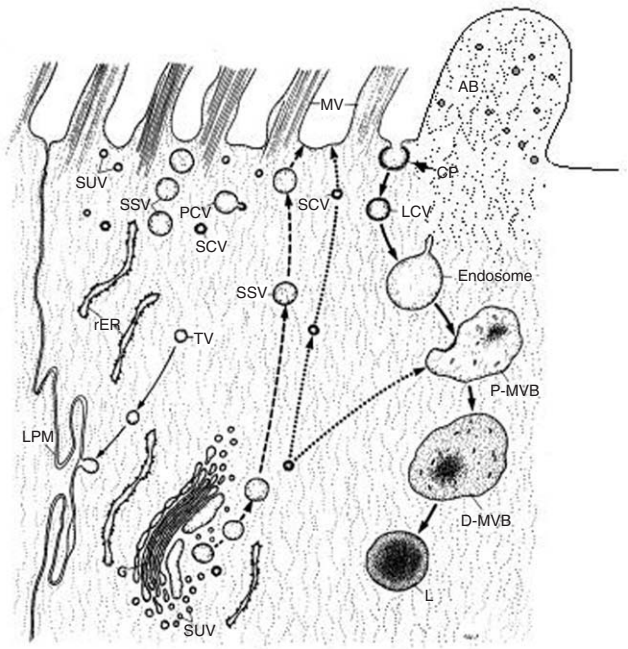

FIG. 7. Schematic representation of the apical and supranuclear cytoplasm of a principal cell of the epididymis. The Golgi apparatus (G) consists of several saccules packed on top of each other, from which the smooth-surfaced secretory vesicles (SSV; 150–300 nm) and small coated vesicles (SCV; 60–70 nm) are derived. The SSV are destined for the apical cell surface with which they will fuse, delivering their content into the epididymal lumen in a regulated manner, by a process termed *merocrine secretion*. The SCV are targeted from the Golgi apparatus to multivesicular bodies (MVBs), delivering their lysosomal contents therein, although some may be destined for the cell surface. The endocytic apparatus consists of coated pits (CP), large coated vesicles (LCV), endosomes, pale (P) and dense (D) MVBs, and lysosomes (L). Tubules emanating from endosomes serve to recycle receptors back to the cell surface. Small uncoated vesicles (SUV; 60–70 nm) are also indicated, some of which may be Golgi derived and involved in constitutive merocrine secretion by fusion with the apical cell surface. An apical bleb (AB) is also indicated and contains mainly polysomes and small vesicles, the origin of which may be Golgi derived. ABs appear to detach from the apical cell surface and upon fragmentation in the epididymal lumen liberate their contents therein for functions involved in sperm maturation. LPM, lateral plasma membrane; Mv, microvilli; PCV, partially coated vesicle; rER, rough endoplasmic reticulum; TV, small vesicles involved in the transcytosis or passage of substances from the lumen to the lateral intercellular space. (Reproduced with permission from Hermo, L., and Robaire, B. [2002]. Epididymis cell types and their function. In *The Epididymis: From Molecules to Clinical Practice* [B. Robaire and B. T. Hinton, Eds.], pp. 81–102. Kluwer Academic/Plenum, New York.).

a variable amount of lipid droplets (Fig. 6) (1,98,100). After injection of tracers into the lumen of the cauda epididymidis, the apical vesicles, endosomes, multivesicular bodies, and lysosomes of clear cells are labeled. This indicates an endocytic role for these cells; this endocytic activity is much greater in clear cells than in the adjacent principal cells, particularly in the cauda epididymidis (100,101). Clear cells normally take up the contents of cytoplasmic droplets released by spermatozoa as they traverse the duct (1,100). Cytoplasmic droplets are formed at the time of the release of spermatozoa and contain Golgi saccular elements that may be involved in modification of the plasma membrane of spermatozoa (102). Clear cells also endocytose a number of different proteins, but often in a region-specific manner (83,87,103,104). These cells become abnormally large and filled with lysosomes after various experimental conditions that disrupt the normal functioning of the testis and epididymis (80).

The acidification of the luminal fluid (see later) is thought to be mediated by clear (and narrow) cells. Indeed, key proteins for this process, H^+-adenosine triphosphatase ([ATPase] or vacuolated [V]-ATPase), carbonic anhydrase II, and soluble adenylate cyclase, are selectively localized to these cells. Further, ClC-5, a member of the voltage-gated ClC chloride channel family, is also expressed exclusively in clear cells and partially colocalizes with the H^+-ATPase in their apical region. Breton's group has proposed that, in clear cells, apical membrane accumulation of V-ATPase is triggered by a soluble adenylate cyclase–dependent rise in cyclic adenosine monophosphate (cAMP) in response to alkaline luminal pH (105–107).

Basal Cells

Basal cells appear in all species studied to date, including humans (1,63,108). Hemispherical in appearance, they adhere to the basement membrane and do not have direct access to the lumen of the duct, although processes of these cells extend at times toward the lumen (Figs. 5 and 6) (109). Like principal cells, basal cells are not thought to divide in adults, nor are they thought to act as stem cells to replenish principal cells (110).

Basal cells possess thin, attenuated processes that extend along the basement membrane from their main hemispherical cell body collectively to cover a large proportion of the circumference of the epididymal tubule (109). However, in rats, after ligation and castration, these cells transform into large, bulbous, dome-shaped cells that are closely packed together and show few short lateral processes (111). Because of the dramatic decrease in size of the epididymal tubules after these treatments, it is likely that the shape and arrangement of basal cells in normal untreated animals is governed in part by the volume and pressure exerted on the tubular epithelium by luminal fluids and sperm derived from the testis. Consistent with this hypothesis is the observation that during postnatal development in the rat, basal cells are transformed from dome-shaped cells to flattened cells that exhibit processes as spermatozoa and

fluids arrive in the corpus and cauda epididymidis by days 49 and 56, respectively (108).

Basal cells possess coated pits on the plasma membrane face opposing the basement membrane and overlying principal cells, suggesting the receptor-mediated endocytosis of factors derived from the blood or principal cells. Basal cells also show an accumulation of a secretory material in Golgi saccules, and distinct secretory granules appear next to the Golgi apparatus (L. Hermo and B. Robaire, unpublished results), as seen in other typical secretory cells (112). The destiny of the secretory material may be to regulate principal cell function or enter the circulation for functions as yet to be determined. Basal cells have been shown also to express apolipoprotein E and alcohol dehydrogenases (113).

It has also been proposed that basal cells may have a role as immune cells because of their ability to respond in number and macrophage antigen expression to the presence of sperm autoantigens in the lumen (114), and it has been postulated that these cells may have an extratubular origin (115).

Studies from Wong's group [reviewed in Leung et al. (116)] have proposed an additional function for basal cells; they suggest that these cells may have a role in regulating electrolyte and water transport by principal cells. This process is proposed to be mediated by the local formation of prostaglandins (PGs) and require the participation of the transient receptor potential (Trp) proteins. The latter serve as transmembrane pathways for Ca^{2+} influx, whereas cyclooxygenase-1 (COX-1) is a key enzyme in the formation of PGs. Both of these proteins are exclusively expressed in basal cells.

Halo Cells

Halo cells are small cells with a narrow rim of clear cytoplasm that are present throughout the epididymal epithelium (Fig. 6) (1). These cells are usually located at the base of the epithelium and contain variable numbers of dense core granules. Halo cells have been described either as lymphocytes (1) or monocytes (117); these two cell types are difficult to distinguish by light microscopy because of their similarity in size and nuclear morphology. Although the exact nature of halo cells has been controversial since they were first described by Reid and Cleland (1957) (57), studies by Flickinger et al. (1997) (118) and Serre and Robaire (1999) (119) have resolved this issue by immunolabeling the main types of immunocompetent cells. It is now clear that, in young adult animals, halo cells consist of helper T lymphocytes, cytotoxic T lymphocytes, and monocytes, but not B lymphocytes. With age, there is a region-specific increase in the number of each of these immune cell types, as well as the occasional appearance of eosinophils (96) and B lymphocytes. In the epididymal epithelium of young rats, the number of cells that stain for antibodies against monocytes–macrophages (ED1+), helper T lymphocytes (CD4+), and cytotoxic T lymphocytes (CD8+) is equivalent to the number of halo cells (119), suggesting that halo cells are, under normal conditions, the primary immune cell in the epididymis.

The Blood–Epididymis Barrier

Given the presence in spermatozoa of proteins that are recognized by the body as foreign, it stands to reason that there should be a continuation beyond the testis of a functional barrier. The probable existence of a blood–epididymis barrier was discussed as early as 1976 (120), and several reviews describing different aspects of the barrier have appeared (121–123).

Structure

The junctional complex between adjacent epididymal principal cells is composed of apically located gap, adherens, and tight junctions. Tight junctions between adjacent principal epithelial cells at their luminal surface form the blood–epididymis barrier (122,124), whereas gap junctions allow communication between adjacent principal cells. These tight junctions form a continuous zonule around the cell, sealing the spaces between the epithelial cells, so that the luminal compartment and the intercellular spaces become separate physiological compartments (125). The tight junctions begin to form at the time of differentiation of the Wolffian duct (126). Using lanthanum nitrate as an electron-opaque tracer that is blocked at tight junctions, the postnatal development of the blood–epididymis barrier was shown to be gradual; its formation is virtually complete by postnatal day 21 in rats (125).

Electron microscopic changes in the structure of the junctional complex of the initial segment have been observed compared with the other segments of the epididymis. In the initial segment, the tight junctions span a considerable length of the apical plasma membrane and have few desmosomes (122). With progress toward the caudal end of the epididymis, a general decrease in the number of tight junctional strands is noted; the span of merging plasma membranes is considerably reduced, but numerous desmosomes are found in the apical region (122,126).

Junctional Proteins

Adherens junctions form a continuous belt and hold neighboring cells together through a family of

calcium-dependent cell–cell adhesion molecules called *cadherins*, which mediate calcium-dependent homotypic interactions (127). The cadherins have also been implicated in the formation and maintenance of tight junctions (127–133). The cytoplasmic domain of cadherins forms a tight complex with several proteins, which either link cadherins to the cytoskeleton or are involved in signal transduction pathways. These include catenins, actinin, vinculin, and zonula occludens-1. The cadherin–catenin complex is essential for cadherin-mediated cell adhesion. Cyr et al. (1992) (134) reported the presence of E-cadherin and P-cadherin messenger RNA (mRNA) in the rat epididymis. Electron microscopy with immunogold labeling indicates that E-cadherin is localized in the extracellular space between the lateral plasma membranes of adjacent principal cells at the level of apical junctional complexes in the adult rat epididymis (122,135,136), as well as in the deeper underlying regions of the extracellular space between the lateral plasma membranes. Similar observations were noted in the human and mouse epididymis (137–139). The composition of the catenin-adhering junctional family of proteins and their relationship with cadherins remain to be established in the epididymis; however, in one study it was shown that, in the normal adult rat epididymis, there was immunostaining for three anti-catenin antibodies (alpha-, beta-, and p120ctn) along the lateral plasma membranes between adjacent epithelial cells (140).

In addition to adherens and tight junctions, the epididymal junctional complex also contains gap junctions (124,141). Gap junctions, made up of proteins termed *connexins*, mediate communication between cells by allowing small molecules to pass from cytoplasm to cytoplasm of neighboring cells, thereby metabolically and electrically coupling them (142). Connexin subunits oligomerize in the trans-Golgi network to form hemichannels or connexons. In the epididymis, gap junctions containing connexin 43 were first localized between principal and basal cells (143). Using a reverse transcriptase-polymerase chain reaction (RT-PCR) strategy, Finnson and Cyr (unpublished observations) have identified at least seven different connexin transcripts in the rat epididymis. Although the presence of multiple connexins in specific cell types is not unique to the epididymis, the large number of different connexins is suggestive of complex communication between epididymal cells.

Functions

The composition of epididymal luminal fluid is distinctly different from that of blood plasma. The blood–epididymis barrier keeps the two fluids in separate compartments (144). The blood–epididymis barrier also maintains a specialized luminal microenvironment for the maturing spermatozoa by restricting the passage of a number of ions, solutes, and macromolecules across the epididymal epithelium (121,144). For instance, molecules such as inositol and carnitine can be concentrated ten- to 100-fold in the lumen of the caput epididymidis, whereas others, such as inulin, L-glucose, and bovine serum albumin, are effectively excluded [reviewed in Robaire and Hermo (1) and Turner (85)]. The blood–epididymis barrier carefully controls the microenvironment so that the spermatozoa are bathed in an appropriate fluid milieu at each stage of maturation as they travel through each segment of the epididymis (85).

This barrier also serves as an extension of the blood–testis barrier. Spermatozoa are immunogenic; they contain proteins on their surfaces that would be recognized as foreign if they were to leave the epididymis (1). The exact function of the blood–epididymis barrier in protecting spermatozoa from the immune system is unclear at this time. The barrier prevents the passage of spermatozoa between epithelial cells, but cellular elements of spermatozoa can be taken up by epithelial cells. However, additional studies are needed to clarify whether, in the adult, any epididymal epithelial cell is capable of acting as an antigen-presenting cell.

Although it would appear that the blood–epididymis barrier is resistant to some foreign substances [e.g., gossypol (144a), estradiol, (144b)], administration of cyclophosphamide to efferent duct–ligated rats resulted in the production of damaged spermatozoa, suggesting that this drug could enter the epididymal lumen and modify spermatozoa. However, very little is known about the role played by this barrier in protecting spermatozoa from toxic substances and immunoglobulins (145). The inability of this barrier to maintain its tightness under conditions of stress, such as aging (136), may play help explain some of the deleterious effects of stressors on sperm function and fertility.

FUNCTIONS TAKING PLACE IN THE LUMINAL COMPARTMENT

The four main functions of the epididymis are transport of spermatozoa, development of sperm motility, development of sperm fertilizing ability, and the creation of a specialized luminal environment conducive of the maturation process through the absorptive and secretory activities of the epididymal epithelium.

Transport of Spermatozoa

Once released in the lumen of the seminiferous tubule, spermatozoa are transported through the

efferent ducts and begin their journey down the epididymis. Several methods have been used to measure the duration of this transit. The most direct method is to incorporate a labeled isotope into the DNA of germ cells at the spermatogonia and preleptotene spermatocyte stages and follow the progression of the first wave of labeled spermatozoa down the epididymis. This approach gives the minimal time required for sperm passage through the epididymis. Total transit time, or transit through each epididymal segment, may also be estimated from the ratio of epididymal sperm reserves and daily testicular sperm production (146), assuming that there is no sperm resorption and no difference in transit speed between segments. Despite some minor discrepancies between estimates obtained with these two techniques, it appears that, regardless of the size of the animal, its sperm production, or its epididymal sperm reserves, the minimal time required for spermatozoa to transit through the epididymis is approximately 10 days (Fig. 8; Table 1), supplemental material on web site). In most species, the average transit time of the majority of labeled spermatozoa is longer than that of the sperm vanguard [e.g., 14 days versus 8 days in bulls (146)]. As a result, there is some mixing of spermatozoa of different ages in the distal part of the epididymis (147). There are two notable exceptions to the 10-day minimum transit time: in the human and chimpanzee, the first labeled spermatozoa pass through the epididymis in 1 and 2 ± 1 days, respectively (Table 1, supplemental material on web site). These values give the most rapid transit time, but the average transit time is 12 days in humans (148), a value comparable with that found in other species.

When one follows the progression of labeled spermatozoa in the different regions of the epididymis, the transit time through the caput and the corpus is quite similar in all species studied, including humans. Most of the difference among species comes from transit through the cauda epididymidis. Surprisingly, in species where this was tested, frequency of semen collection did not influence transit time through the epididymis markedly (146,149,150). In bulls, daily sperm collection must exceed twice the daily testicular sperm production to lead to an acceleration of sperm transit of only 3 days for the first wave of labeled spermatozoa (146), whereas in rabbits different frequencies of ejaculation ranging from once per week to four times in 1 day had no effect on sperm reserves in the caput and corpus epididymidis, but dramatically reduced sperm reserves in the cauda epididymis and vas deferens (151).

Spermatozoa enter the epididymis propelled by testicular fluid and possibly the beat of the ciliated cells of the efferent ducts. However, in the epididymis, the epithelium is lined by immotile stereocilia and the massive fluid uptake taking place in the ductuli efferentes and the initial segment of the epididymis drastically reduces this fluid flow (152). Transport takes place against an increasing hydrostatic pressure gradient from testis to cauda epididymidis (153), and proceeds even when fluid flow from the testis is prevented by ligation of the ductuli efferentes (16,154). It is unlikely that these mechanisms alone are responsible for sperm transport.

The epididymis is surrounded by a smooth muscle layer of increasing thickness and adrenergic innervation from proximal to more distal regions (155).

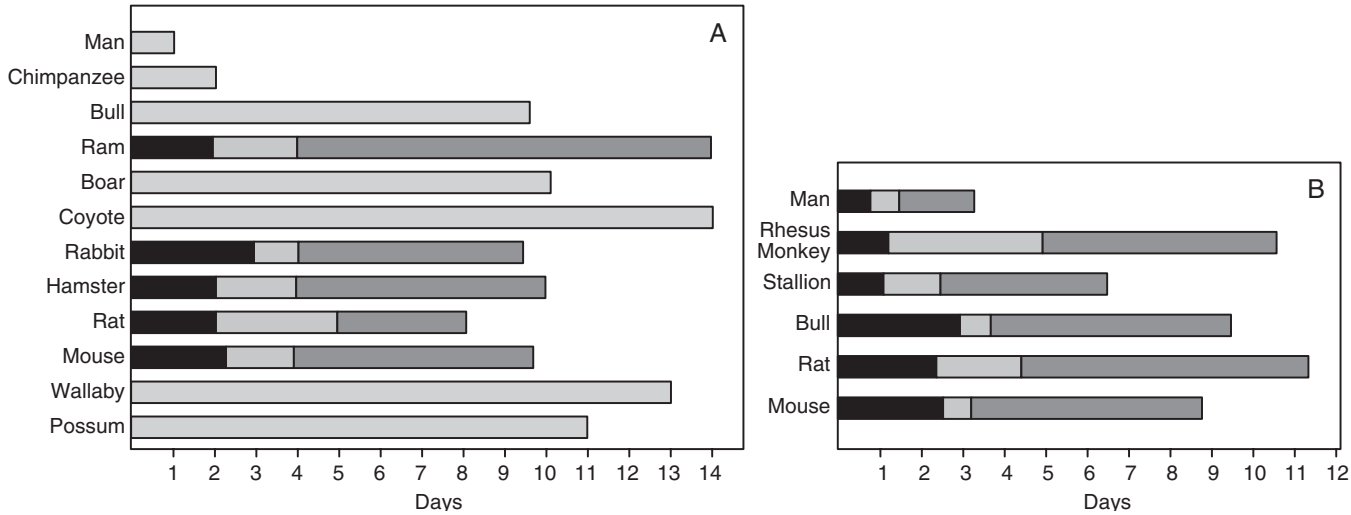

FIG. 8. A: Minimal time for epididymal transit determined by the progression of the first wave of labeled spermatozoa after isotope injection [human (148); chimpanzee (927); bull (146); ram (150); boar (928); coyote (929); rabbit (147); hamster (930); rat (165); mouse (166); tammar wallaby and brushtailed possum (931)]. **B:** Duration of sperm transit through the whole epididymis and each segment determined by the ratio of epididymal sperm reserves to daily testicular sperm production [human (932), (933); rhesus monkey (934); stallion (935); bull (18), (936); rat (937); mouse (166)].

Therefore, the mechanism responsible for driving the contents through the lumen of the resting epididymis has been attributed primarily to the rhythmic muscular contractions of the smooth muscle lining the epididymal tubule (156–159). These elegant studies have shown a relationship between the electrical and contractile activities of the epididymal tubule and the progression of oil droplets injected in the lumen. The droplets do not move in a linear fashion, but back and forth. The movement forward starts when the spread of electrical activity approaches the droplet and stops or reverses when the electrical activity wanes or changes direction. Because the electrical pacemakers are randomly distributed and the electrical pulse spreads in both directions, droplets injected at the same time may spread in the lumen and follow different courses. The net distance covered during each pendular movement is small. Nevertheless, the droplets progress downward towards the vas deferens, where the frequency of electrical activity is lower than in the caput epididymidis (156,158). The rate of luminal flow is not uniform in the different segments of the epididymis (158–160). The progression of droplets decreases from 420 mm/2 hours in the initial segment to 64 mm/2 hours in the distal caput and 25 mm/2 hours in the cauda epididymidis and vas deferens (158). It is likely that the progression of oil droplets mimics that of spermatozoa in the epididymis. In bulls, labeled gold-coated beads injected in the rete testis are grouped in the distal caput epididymidis 2 days after injection and in the cauda 5 days after injection, a transit time comparable with that of labeled spermatozoa, but 6 days after injection, beads are spread throughout the cauda (146). Therefore, the progression of the epididymal luminal content is a dynamic process, resulting in a mixing of luminal content, and is controlled by the electrical and contractile activity of the epididymal tubule.

The smooth muscle contractions of the epididymal tubule and transit of spermatozoa therein are influenced by several factors, both hormonal and neuronal. Castration depletes epididymal sperm reserves (161–163) and increases intraluminal pressure, contractility of the epididymis (164), and sperm transport (165). Testosterone treatment reverses the effect of castration, indicating that androgens control the contractility of the epididymal tubule to ensure an optimal rate of sperm transport. Estrogen, on the other hand, speeds up murine sperm transport drastically from 9.7 to 2.1 days (166).

Contractility of the epididymal tubule is also influenced by PGs (167,168). $PGF_{2\alpha}$ increases the frequency and amplitude of contractions in proximal epididymis tubules in vitro, whereas PGE_2 decreases these contractions (168). The endogenous levels of PGs are consistent with their regulation of basal contractility of the proximal epididymis (168).

Neurohypophysial peptides, such as oxytocin or vasopressin, mediated by receptors present in the epididymis (169–173), also increase epididymal contractility both in vitro (174–176) and in vivo (177–180). In several species, including humans (169), this results in an increase in the number of ejaculated spermatozoa or spermatozoa transported through the epididymis (179,181–187). Although the relative effect of oxytocin and vasopressin may vary among species, depending on the dose of the peptide or on the epididymal segment used in the in vitro studies (180,187), it is clear that neurohypophysial peptides regulate both basal contractility of the epididymis and, on release in the peripheral circulation around the time of ejaculation (188–192), transport of spermatozoa through the vas deferens. Interestingly, the effect of oxytocin on epididymal motility is regulated by estrogens in part by an upregulation of the oxytocin receptor gene and protein (175). This may account for the estrogen-induced accelerated sperm transport (166), and suggests an interplay between steroids and neurohypophysial hormones in the regulation of epididymal contractility.

Neuronal regulation is also involved in epididymal contractility and sperm transport. This was first demonstrated by Simeone in 1933 (193) using surgical sympathectomy and confirmed in later studies using either surgical (168) or guanethidine-induced chemical sympathectomy (194–197). Surgical removal of a single neuronal ganglion, the inferior mesenteric ganglion that provides sympathetic innervation to the cauda epididymidis, is sufficient to slow sperm transport through the epididymis (198). Indeed, adrenergic and cholinergic drugs affect contractility of the epididymis both in vitro (199) and in vivo (179,200–203). Temperature also affects epididymal contractility: a switch from scrotal to body temperature increases the frequency and spread of electrical activity of the smooth muscle of the epididymis (204), and significantly speeds up sperm transport through the epididymis (205), thus having potential deleterious effects on sperm maturation and fertility.

Although a neuromuscular mechanism may not be the only one responsible for sperm transport in the various segments of the epididymis and in all species, it appears to be the main mechanism responsible for sperm transport through the epididymis in mammals.

Maturation of Spermatozoa

Fertilizing Ability

In lower vertebrates, such as cyclostomes, fish, and amphibians, spermatozoa released from the testis are fully motile and competent to fertilize. In contrast,

in higher vertebrates, spermatozoa become functionally mature as they pass through the epididymis. They acquire the ability to move forward when released from the epididymis, ascend the female genital tract, undergo the acrosome reaction, bind to and penetrate the egg vestments, and achieve syngamy with the female gamete.

In all species examined thus far, a gradient of fertilizing potential is observed as spermatozoa traverse the epididymis (Fig. 9). Although there is some species variation with respect to the exact site at which spermatozoa first gain their fertilizing potential, it is clear that to be competent to fertilize in vivo, spermatozoa leaving the testis have to pass through some part of the proximal epididymis.

This fertility profile can be altered somewhat using in vitro fertilization (IVF) techniques that bypass some or all steps in the normal fertilization process. For example, rabbit spermatozoa from the caput epididymis, which do not fertilize more than 2% of eggs after in vivo insemination (20), fertilize 8% of eggs after in vitro insemination (206). A similar shift in fertility profile is observed when mouse spermatozoa are inseminated in vivo, in vitro, or after subzona injection (Fig. 10). Testicular spermatozoa injected directly into the oocytes can even achieve a fertilization rate of 94% (207). Fertilization also can be achieved when round spermatids are injected or electrofused with oocytes (208), or when secondary or

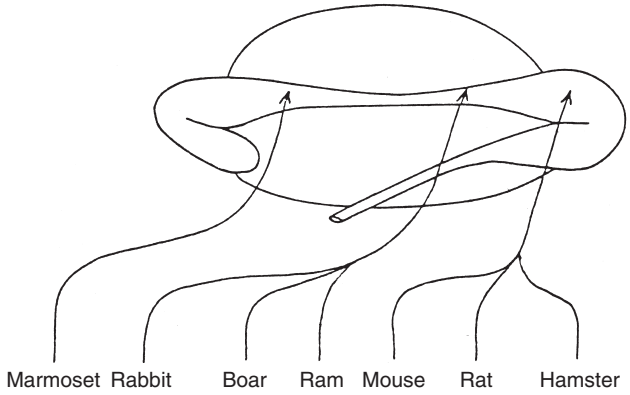

FIG. 9. Approximate site in the epididymis where spermatozoa acquire their fertilizing ability. [Information for each species, as brought together by Orgebin-Crist (938), was obtained from the following sources: marmoset (939), rabbit (20,210,940) boar (941), ram (942), mouse (943,944), rat (161,211,244), and hamster (945–948).]

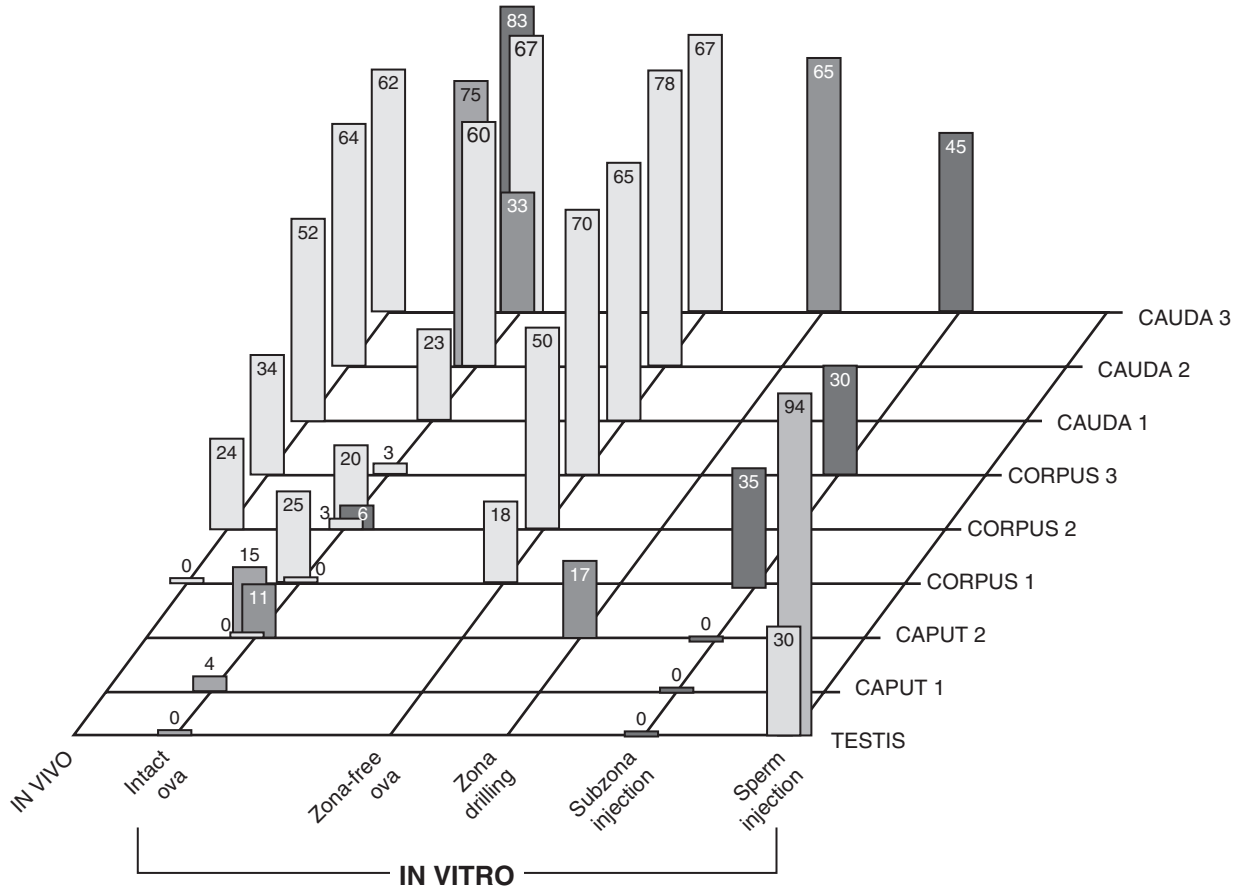

FIG. 10. Fertilizing capacity of mouse spermatozoa from successive segments of the epididymis inseminated either in vivo (943) or in vitro with cumulus-encased or cumulus-free ova (218), with intact ova (943,944), with zona-free ova (944), after zona drilling (217), or by subzonal sperm injection (949).

even primary spermatocytes are injected into the ooplasm (207,209). However, the fertilization rates are lower than after injection of testicular spermatozoa.

Moreover, it appears that the acquisition of fertilizing potential is not a simple "on" or "off" state. Initial studies with rabbits (20,210), rats (211), and rams (212,213) showed that spermatozoa first gain the ability to fertilize eggs, and only after further transit through the epididymis acquire the ability to produce complete litters of viable offspring. Orgebin-Crist noted an increase in both preimplantation and postimplantation loss when rabbits were inseminated with corpus versus ejaculated spermatozoa (20); this is apparently due to a delay in the fertilization of eggs (20,214) and in the first zygotic division (215). Although Overstreet and Bedford (216) were unable to confirm these observations, more recent studies in mice (217) reported that only 8% of oocytes fertilized by caput epididymal spermatozoa were capable of developing into blastocysts in vitro, compared with 48% of oocytes fertilized by cauda spermatozoa; Lacham-Kaplan and Trounson (218) confirmed that a high rate of embryonic arrest and retarded development occurs in mouse oocytes fertilized by epididymal spermatozoa that have just gained their fertilizing capacity. Although testicular spermatozoa and spermatids fertilize 94% and 37% of eggs, respectively, after injection into the oocytes, only 54% and 28%, respectively, of the fertilized eggs develop into live offspring when transferred into foster mothers (207). Collectively, these experiments show that passage through the epididymis endows spermatozoa with the ability to ascend the female genital tract and interact with the egg. This maturation process can be circumvented because spermatid-injected oocytes develop into live offspring, but the chances of normal development are higher after injection of more mature spermatozoa.

The experimental protocol of insemination of equal numbers of spermatozoa from succeeding segments of the epididymis from one individual is obviously not possible in humans. However, in cases of obstructive azoospermia, epididymovasostomies have been done to recanalize the epididymis. The cumulative results show a low pregnancy rate when the anastomosis is done proximally and an increased incidence of pregnancies with more distal connections (219,220). These results, although not directly comparable with the animal studies, imply that in human, as in other species, there is a progressive maturation of sperm fertilizing ability in the epididymis. They also suggest that the fertility profile may be shifted more proximally compared with other species. Pregnancies have been achieved even after reanastomosis of the efferent ducts to the vas, but the postoperative interval before pregnancy occurred was longer (2 years) (221) than after corpus–vas anastomosis (6 to 13 months) (220). If spermatozoa from the efferent ducts had the same level of maturity as spermatozoa from the corpus, one would expect the same postoperative interval, but the small number of cases precludes any generalization.

In cases of obstructive azoospermia or congenital vas agenesis, spermatozoa can also be aspirated from the epididymis and inseminated in vitro (IVF). There is a statistical difference in fertilization rates with spermatozoa retrieved from different levels of the epididymis (222), confirming the progressive maturation of human spermatozoa in the epididymis observed after reanastomosis (219). After IVF and transfer, pregnancies have been reported with spermatozoa recovered from the corpus epididymidis (223–225), and even the proximal caput epididymidis (226,227). However, the consequences of either obstructive azoospermia or congenital vas agenesis on the structure and functions of the epididymal epithelium have not been investigated.

If immature human spermatozoa bypass normal sperm ascent in the female genital tract and sperm–egg interaction, and are injected directly into the ooplasm (intracytoplasmic sperm injection [ICSI]), they can form zygotes. As in other species, the overall fertilization rate is higher than after conventional IVF (45% versus 6.9%) (228). Even testicular spermatozoa achieve fertilization rates of approximately 50%. Nevertheless, in all studies, there is a small but consistent difference in fertilization rates after injection of testicular and epididymal ejaculated spermatozoa (229–236). In all studies but one (234) that reported statistical analyses, the differences in fertilization rates were significant.

Delayed fertilization and cleavage arrest were reported in one case of IVF with spermatozoa from the corpus epididymidis (224). In a large series (236) comparing outcome after ICSI with testicular, epididymal, and ejaculated spermatozoa, testicular spermatozoa had not only statistically significantly lower fertilization rates (53.4%) than epididymal (58.5%) or ejaculated spermatozoa (64.8%), but statistically lower yields of good-quality embryos (47%, 40%, and 59%, respectively). The embryos chosen for transfer yielded a similar pregnancy rate per transfer (23.1%, 31.3%, and 27.4%, respectively). The congenital malformation rate and the developmental outcome of children born after ICSI with testicular, epididymal, or ejaculated spermatozoa are similar to that of children born after conventional IVF (237–239) or in the general population. Collectively, these clinical studies indicate that, as in other mammalian species, human sperm capacity for optimal fertility in vivo increases during epididymal transit, and procedures bypassing critical steps of the natural process of fertilization

permit fertilization with immature spermatozoa. However, unlike other species, the fertility profile of human epididymal spermatozoa appears to be shifted more proximally because pregnancies have been reported with spermatozoa from the proximal epididymis.

Motility

Concomitant with the acquisition of fertilizing ability, a number of sperm characteristics undergo maturational changes. The first one to be recognized last century, by Tournade (8), was the epididymal maturation of the sperm potential for motility. This was subsequently confirmed in numerous species of higher vertebrates, from lizard (240,241), to mouse (242), rat (211,243–251), hamster (252,253,253a), guinea pig (254,255), rabbit (20,251,256,257), boar (258,259), goat (260), ram (261–263), bull (264,265), monkey (266–268), and human (75,269–271). The acquisition of motility is observed whether the testis and epididymis are located in the scrotum or in the abdomen, as in the elephant (272), the hyrax (272,273), and the armadillo (273). The maturation of sperm motility potential involves both a quantitative increase in the percentage of motile spermatozoa and a qualitative difference in motility pattern. Testicular spermatozoa are either immotile or display only a faint twitch of the flagellum. Spermatozoa released from the caput epididymidis swim in a circular pattern, whereas spermatozoa released from the cauda move progressively and vigorously forward.

Other Maturational Changes

In addition to the capacity for progressive motility, epididymal spermatozoa develop the capacity to undergo the acrosome reaction [mouse (274), ram (275), pig (276), dog (277), monkey (278), and human (279)], recognize and bind to the zona pellucida [mouse (280), pig (276)], and fuse with the vitelline membrane as tested with zona-free hamster eggs [pig (281), human (281a,282)]. Concomitant with these functional changes, spermatozoa undergo structural changes during epididymal transit: migration of the cytoplasmic droplet along the sperm flagellum, acrosomal reshaping, changes in the sperm nuclear chromatin and some tail organelles, and changes in the sperm plasma membrane [reviewed in Bedford (283,284)]. Collectively, these changes underpin the functional maturation and subsequent storage of spermatozoa in the epididymis, but none of these changes has been shown to be the only determinant of the acquisition of fertilizing ability.

Regulation of Sperm Maturation

Benoit (12) demonstrated that the function of the epididymis, and the maturation and survival of spermatozoa within it, depended on hormones secreted by the testis. After bilateral castration, not only did the epididymal epithelium dedifferentiate, but epididymal spermatozoa died quickly, whereas after unilateral castration spermatozoa maintained their potential for motility on the castrated side 2 months after the operation. Experiments conducted by Bedford (19) and Orgebin-Crist (21,285) showed that although immature rabbit spermatozoa develop the capacity for motility and fertility when retained by ligatures in the proximal corpus, they do not become fertile when retained in the caput, although they develop a mature pattern of forward motility. This indicates that sperm maturation is a multistep process and shows that although the development of a mature pattern of motility is necessary, it is not sufficient to render a spermatozoon capable of fertilizing. It also shows that whereas some of the maturational changes, like motility, may be intrinsic to the sperm cell and develop with time, others, such as the ability to interact with the egg, depend on the epididymal environment. The latter is conditioned by testicular androgens because sperm maturation occurs after castration, hypophysectomy, or in organ culture only when androgen is administered in vivo or included in the culture medium (161,286–288).

Studies of coincubation of epididymal spermatozoa with epididymal epithelial cell cultures have confirmed the role of the epididymis in promoting sperm maturation. In such cultures, brushtail possum and tammar wallaby spermatozoa from the proximal caput underwent the morphological maturational change in head orientation accompanied by the development of progressive motility, normally only observed in vivo (289,290). In hamsters, mice, and humans, coculturing of immature spermatozoa from the caput or corpus epididymis with cauda cells not only increased their motility (291–293), but increased their capacity to bind to salt-stored zona pellucida (293), to fertilize (291,292), and to support the development of embryos (sired by corpus spermatozoa) (292). These maturational changes are promoted by androgen-dependent factors from epididymal principal cells because cocultures maintained in the absence of androgens fail to induce maturation (292,293). The overwhelming evidence from all these experimental studies, both in vivo and in vitro, is that the final stages of the sperm maturation process in all mammalian species studied to date, including humans, depend on the epididymis.

However, reports of human pregnancies in two cases where the vasa efferentia was anastomosed to

the vas deferens, thereby bypassing the entire epididymis, led to a challenge to the concept that the epididymis is necessary for the acquisition of fertilizing ability (221). As discussed previously, subsequent reports noted that the more distal the anastomotic site, the greater the chances of fertility, confirming that the epididymis provides the milieu necessary for optimal fertility. Nevertheless, Temple-Smith (294) revisited the issue in elegant experiments using epididymovasostomies at different levels of the epididymis in rabbits and rats to assess the fertility of these animals. In both of these species, anastomosis of the distal initial segment to the vas, bypassing the distal caput, corpus, and cauda epididymis, resulted in a significant reduction in sperm viability, motility, and fertility, confirming that spermatozoa require exposure to the epididymal environment for normal development of fertilizing ability. Interestingly, in rats, bypass of the distal caput and proximal corpus, either by epididymo-epididymostomy or graft of a vas bridge, had no effect on sperm motility, viability, or fertility. These elegant studies show clearly that the epididymis plays a major role in the post-testicular maturation of spermatozoa, but they also show that exposure to the initial segment may be sufficient or that the lack of exposure to the epididymal regions where spermatozoa normally become fertile may be compensated by secretions from other epididymal regions. As discussed later, epididymal spermatozoa are exposed to a highly complex environment, but the nature of the changes induced by epididymal transit that are required to promote optimal fertilization remains to be established.

Storage of Spermatozoa

The major site for storage of spermatozoa in the excurrent duct system of mammals is the cauda epididymidis. Although normal transit time in mammals through the cauda epididymidis is in the range of 3 to 10 days (Fig. 8; Table 1, supplemental material on web site), spermatozoa can be stored in this tissue for periods extending beyond 30 days (295); in bats, spermatozoa may be stored in this tissue for many months and retain their function (296). With storage in the cauda epididymidis, a loss in fertilizing ability was found to occur before a loss in motility (297). Interestingly, spermatozoa that were aged in the male reproductive tract of rabbits, presumably in the cauda epididymidis, induced a tenfold higher incidence of chromosomal abnormalities in the resulting blastocysts than did their fresh counterparts (298).

Based on studies in a number of mammalian species [reviewed in Amann (299)], it was noted that 50% to 80% of spermatozoa present in the excurrent ducts were found in the cauda epididymidis and that approximately 50% of these spermatozoa were available for ejaculation (299). When animals (rabbits, stallions, and bulls) were at sexual rest (i.e., 7 or more days without ejaculation), the number of stored spermatozoa that were available for ejaculation was three- to fivefold greater than the daily sperm production rate and two- to threefold greater than that found in a "typical" ejaculate; frequent ejaculation, that is, one ejaculate every 1 to 2 days, did not result in a change in sperm production rate but did markedly decrease caudal epididymal sperm reserves and the number of spermatozoa in the ejaculate (299). In contrast, the human, whose sperm production rate is well below that of most other mammals, has a sperm reserve that is only approximately equal to the number of spermatozoa found in an ejaculate, whether at sexual rest or not (299).

In a series of studies on the evolution of the scrotum, Bedford proposed that the prime moving force behind the formation of the scrotum was the need to store spermatozoa at a low temperature (205,300,301); he suggested "that migration to a scrotal site has been ordained primarily by the sperm storage region—the cauda epididymidis—and that the function of the testis has only been an incidental factor in this evolutionary development" (301). This proposal was based on anatomical observations and on some physiological studies where the epididymis was placed abdominally while the testis remained in the scrotum; the rate of sperm passage through the cauda epididymidis was found to increase approximately twofold in the rabbit after such a procedure.

One would expect that the luminal environment required for the storage of spermatozoa in the cauda epididymidis versus that required for the acquisition of fertilizing ability would be different. Although many differences have been described in the luminal makeup of ions, small organic molecules, proteins, and glycoproteins between the cauda and the rest of the epididymis (see later), those special conditions that allow for the storage of spermatozoa in a quiescent state for long periods in this tissue have not yet been elucidated, although changes in pH (302) in several species and the presence of immobilin in the rat and hamster (302,303) have been proposed to be major factors.

Although it is clear that the major function of the distal segments of the epididymis is to store mature, live spermatozoa, other functions have been ascribed to this tissue. Abnormal-appearing or dead spermatozoa have often been seen in the cauda epididymidis (304,305), and the ability to recognize such spermatozoa and then develop a mechanism to neutralize or destroy them has been proposed recently by several groups.

Sutovsky et al. (2001) (306) proposed that the cell-surface ubiquitination of defective spermatozoa supplies the necessary signal for these cells to be phagocytosed by epididymal principal cells, thus providing a mechanism for sperm quality control. However, based on extensive histological observations of the normal epididymal epithelium and the rarity with which sperm phagocytosis is observed, it is apparent that phagocytosis of spermatozoa is an unlikely mechanism for the disposal of the large number of spermatozoa produced daily (307). If a quality control system is present in the epididymis, then it is likely to be more subtle than the phagocytosis of marked spermatozoa.

Studies from Olson's group (308,309), have identified a fibrinogen-like protein (fgl2) that is secreted selectively in the proximal cauda epididymidis of the hamster and that binds to and coats nonviable, but not viable, luminal spermatozoa. Unlike the hypothesis of Sutovsky's group, these investigators propose that the epididymis possesses a specific mechanism to identify and envelop defective spermatozoa with a protein complex containing fgl2 that remains within the epididymal lumen. Both of these novel mechanisms require that epididymal epithelial cells have the ability somehow to recognize and tag defective spermatozoa. Although conceptually appealing, more extensive studies using different species and means of creating "abnormal" spermatozoa, such as drug treatments, are required to establish this as a function of the normal epididymis.

In a review, Jones (2004) (310) has hypothesized that after androgen withdrawal, either by orchidectomy or as a result of seasonal variation in seasonal breeders, a death pathway is activated that leads to the dissolution of spermatozoa. Such a mechanism would ensure that the epididymis is cleansed of defective and dead spermatozoa in preparation for the next breeding period. How androgen withdrawal would activate such a pathway remains to be determined, but it is interesting to note that many genes are activated in the epididymis after orchidectomy (311).

Protection of Spermatozoa

It is clear that the blood–epididymis barrier allows for the production of a specialized luminal fluid microenvironment that is important for sperm maturation. However, another critical role played by this barrier is protection of the maturing spermatozoa. In addition, a series of elaborate defense mechanisms that help to protect spermatozoa from the immune system, harmful xenobiotics, and reactive oxygen species (ROS) have been developed in the epididymis. The defense mechanisms include restricting the types of compounds that can enter the epididymal lumen, the synthesis and secretion of specific proteins such as the defensins and defensin-like molecules, rapid elimination of potential harmful agents through the synthesis and secretion of antioxidant and conjugating enzymes, and the synthesis and secretion of antioxidant compounds such as glutathione and taurine. Because spermatozoa mature in a hyperosmotic environment, the epididymis also ensures that the spermatozoa are protected from potential rapid changes in osmolality and can regulate their cell volume.

It was shown some 50 years ago that spermatozoa are highly susceptible to oxidative damage and that hydrogen peroxide was responsible for loss of motility and cell death (312,313). Later, through a series of well-designed studies, Jones and Mann clearly showed that when spermatozoa were incubated under aerobic conditions, they produced an organic peroxide and released a substance that was believed to be a lipid (314–317). When incubated in the presence of lipid peroxides, spermatozoa became irreversibly immotile and released numerous intracellular enzymes. From this series of studies, it was suggested that incubation of spermatozoa under aerobic conditions resulted in lipid peroxidation by peroxides or lipid radicals that were responsible for structural damage, loss of motility, decline in metabolic activity, and release of intracellular enzymes. Because mammalian spermatozoa have a high content of polyunsaturated fatty acids in their membranes, they are highly susceptible to lipid peroxidation by ROS (317–320). It is crucial that spermatozoa be protected from the deleterious effects of ROS as they progress along the epididymal duct; lipid peroxidation of membranes has been correlated with midpiece defects (321), decreased motility due to axonemal defects, and reduced intracellular ATP levels (322–324), as well as impaired capacity for fertilization [reviewed in Vernet et al. (2004) (320)]. Spermatozoa are protected from ROS by superoxide dismutase (325–328). This enzyme protects spermatozoa from lipid peroxidation by ROS through dismutation of the reactive oxygen to hydrogen peroxide and water. Hydrogen peroxide is then rapidly converted to water by the enzymes catalase or glutathione peroxidase (328,329,330).

In a continuing effort to prevent the oxidative damage of spermatozoa, the epididymis has developed an elaborate system to ensure that spermatozoa are protected as they mature along the epididymal duct. Each region or segment of the epididymis has developed its own sperm protective mechanisms primarily because (a) the metabolic activity differs from one

region to the next, thereby producing different ROS species that need to be eliminated appropriately; and (b) spermatozoa are in a different state of maturity in each region, and therefore differ with respect to susceptibility to oxidative damage. For example, spermatozoa need to be especially protected from ROS as they enter the initial segment. The luminal fluid in this region is highly oxygenated (331), the epithelial cells are surrounded by a dense capillary network (332), with blood flow exceeding that of the distal epididymal regions (333,334), and the initial segment cells are highly metabolically active [reviewed in Hinton et al. (335)]. This unique combination results in the generation of ROS from several sources, including the endothelial and initial segment epithelial cells [reviewed in Vernet et al. (320)]. Therefore, it is not surprising that the initial segment expresses antioxidant enzymes. In the distal epididymal regions, the epithelial cells are still metabolically active, and the spermatozoa are continually exposed to an oxygen-rich environment; however, there is less vascularization compared with the initial segment. Hence, spermatozoa are still subjected to ROS production, but the type of ROS may well be different from the types produced in the initial segment. Hence, the distal epididymal regions have alternative strategies to protect spermatozoa.

The major antioxidant enzymes present in the epididymis (320) include superoxide dismutase (336,337), γ-glutamyl transpeptidase (338–344), glutathione peroxidases (345–349), glutathione transferases (109,350–354), and indolamine dioxygenase (355). In addition, the lumen of the epididymis contains antioxidant molecules such as glutathione, taurine, and tryptophan as the substrate for indolamine dioxygenase. Each of these antioxidant enzymes and molecules is found to varying degrees throughout the length of the epididymis.

Hence, the epididymis plays a critical role in the protection of spermatozoa from oxidative stress and harmful xenobiotics. The processes by which the epididymis protects spermatozoa have also been considered to be a prime target for the development of a male contraceptive (320).

Microenvironment for Maturation, Protection, and Storage

From the previous discussion it can be seen that the epididymis plays an important role in sperm maturation. Although the actual time spent in the epididymis may be important for the maturation process, spermatozoa must still undergo considerable remodeling and be protected and stored in a specialized luminal fluid milieu. Crabo (1965) (152) first recognized the importance of the epididymal microenvironment: "The consensus today is doubtless that the epididymis actively promotes maturation of spermatozoa. The natural way for the epididymis to exert this influence would be via regulation of the sperm cells' environment, i.e., epididymal plasma: the environment in the caudal portion should favor maximal survival time of the spermatozoa which may be stored there for relatively long periods, without losing their fertilizing power and motility." In view of several published comprehensive reviews on the formation of the epididymal luminal microenvironment (144,356–361), this section summarizes the contributions of ions, organic solutes, and proteins to the specialized luminal fluid milieu. Although much is known concerning the composition of the epididymal luminal fluid, more studies are needed to understand the precise contribution of each component to sperm maturation.

Perhaps the most underappreciated role of the epididymis is the manner by which it forms the microenvironment. To accomplish this formidable task, the epithelium must be structured in a way such that (a) it prevents and regulates the entry of blood-borne substances into the lumen; (b) it has the ability to synthesize, secrete, and absorb components; (c) it is arranged so that spermatozoa come into contact with the appropriate environment at the appropriate time—this is the most challenging aspect to understand because it implies that each cell along the duct knows precisely what its neighbors are doing; and (d) it can respond to spermatozoa, which in turn regulate the microenvironment. Hence, there is a series of complex and ever-changing interactions between the epididymal epithelium, the microenvironment, and spermatozoa from the beginning to the end of this tube that is several meters in length.

Water Movement and Its Influence on the Formation of the Luminal Microenvironment

Before discussing the composition of the luminal fluid microenvironment, it is important to consider water movement. The efferent ducts and the epididymis are water-transporting epithelia. Hence, the concentrations of ions, organic solutes, and proteins depend on the movement of water into and out of the lumen. Thus, the measured concentrations of any ion, solute, or protein in epididymal luminal fluid are not only due to direct secretion into the lumen but also to the amount of water that is being reabsorbed. Water movement out of the duct is quite extensive.

For example, the water-transporting ability of the rat efferent ducts is 5 to 10 times less than that of the proximal kidney tubule, but the water-transporting rate of the efferent ducts of the quail almost matches that of the rat nephron (356). Remarkably, 70% to 96% of the fluid leaving the testis is reabsorbed in the efferent ducts in several species; for example, 96% is reabsorbed in rat (362), 90% in boar (152), 87% in the tammar wallaby (363), and 96% in the elephant (364), with concomitant declines in sodium and chloride concentrations but increases in potassium and hydrogen ion concentrations from the rete testis to the caput region. Within 24 hours of efferent duct ligation in the rat, there is a remarkable increase of nearly 50% in testicular weight due to accumulated fluid, thus demonstrating how effective the efferent ducts and epididymis are at removing large volumes of water (365). From the caput region onward, 95% of the remaining 5% to 10% water is reabsorbed, ultimately increasing the concentration of spermatozoa from approximately 10^4/mL in rete testis to 10^9/mL in the cauda epididymidis.

Hence, when analyzing the concentration of various ions, solutes, and proteins, it is important to take into account the reabsorption of water. For example, the increase in the concentration of some organic solutes seen from the caput to cauda epididymidis may not necessarily be due to an increase in the secretion of that solute, but entirely to water movement. Examples of this include carnitine and inositol (366,367). However, if water reabsorption is taken into account when calculating what the total protein concentration should be, a much higher value is calculated compared with the actual value measured (368). This would indicate that considerable protein is being reabsorbed, a well-known role of the epididymis. A simple analysis of this kind may help determine the extent, if any, of movement of ions, solutes, and proteins across the epididymal epithelia from proximal to distal regions. It should be emphasized that the measured concentration of any ion, solute, or protein in the luminal fluid of any epididymal region is a product not only of secretion, absorption, and water reabsorption but of sperm uptake, degradation, and metabolism.

Ionic Microenvironment

Crabo (1965) (152) and Levine and Marsh (1971) (369) were perhaps the first to show that the ionic luminal fluid composition of the epididymal duct is distinctly different from blood plasma. However, through a more thorough analysis of epididymal luminal fluid collected by micropuncture, we now know the exact ionic composition to which spermatozoa are exposed as they progress along the duct (369,370,371). Briefly, spermatozoa move from an environment in the seminiferous tubule that has a pH of approximately 7.3 and lower sodium concentration but higher potassium concentration than blood plasma [reviewed in Hinton and Setchell (372)], to an environment in the initial segment/caput region with pH 6.5 and sodium and potassium concentrations closer to those in blood plasma. Spermatozoa then move into a progressively lower ionic environment from the caput to the cauda but a progressively higher organic solute and protein environment [reviewed in Hinton and Palladino (144), Rodriguez and Hinton (361), and Turner (359,373)]. Sodium, chloride, calcium, and magnesium concentrations decline from the caput to cauda epididymidis, but phosphorus (as phosphate) and potassium increase in concentration (Fig. 11). The physiological importance of the ionic microenvironment is unclear, but it may be involved in the development of sperm motility and in keeping spermatozoa in a quiescent state as they mature (374–377). More studies are needed to test the

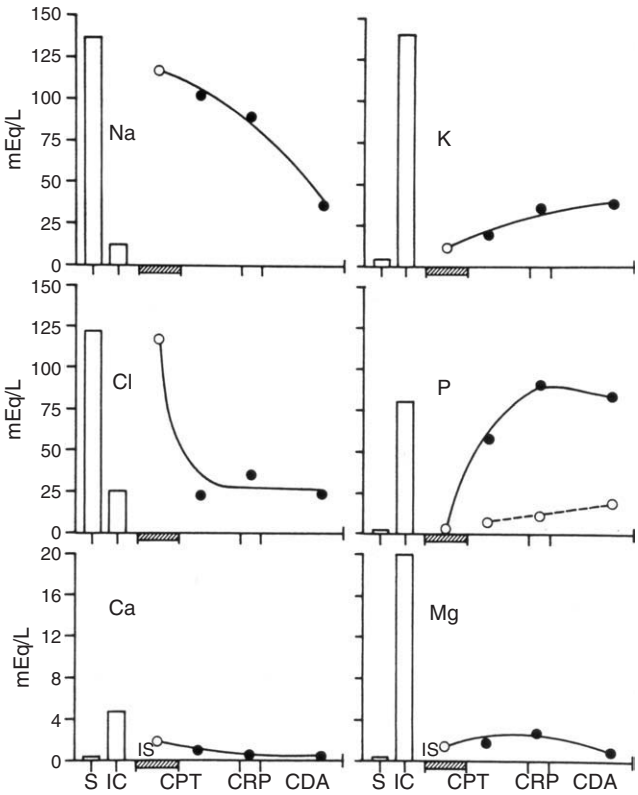

FIG. 11. Serum, intracellular, and intraluminal concentrations of sodium, potassium, chloride, phosphorus, calcium, and magnesium in the rat epididymis. Serum (S) and intraluminal values are from Jenkins et al. (371). Intracellular (IC) values are those expected in typical somatic cells (950) and represent anticipated values in the epididymal epithelial cells (initial segment [IS], caput [CPT], corpus [CRP], and cauda [CDA] epididymidis). Data connected by *hatched lines* in the P panel are P_i data from Hinton and Setchell (951).

hypothesis that ions play a critical role in sperm maturation.

Organic Solute Microenvironment

As well as analyzing the luminal fluid ionic microenvironment, Levine and Marsh (1971) (369) also measured the osmolality of fluid collected from each epididymal region and then compared these values with the sum of osmotically active ions at each region. They noted a difference. This osmotic difference increased from the caput to cauda epididymidis to reach over 250 mOsm/kg water; the investigators suggested that this difference was due to the presence of organic solutes. Later studies by several groups showed that this was indeed the case, with the identification of solutes such as glutamate, taurine, L-carnitine, myoinositol, glycerylphosphorylcholine, phosphorylcholine, sialic acids, and many other amino acids present in different concentrations through the epididymal duct of many species, including the human [reviewed in Turner (85,359), Hinton and Palladino (144), and Rodriguez and Hinton (361)].

The total concentration of organic solutes in the luminal fluid of the rat testis and epididymis increases from approximately 10 to 20 mM in the seminiferous tubule to 100 to 150 mM in the caput and to over 200 mM in the cauda epididymidis. Although virtually nothing is known about the roles of each solute, evidence suggest that they may play a critical role in the protection of spermatozoa and the epididymal epithelium from osmotic stress, similar to the role played by these solutes in the kidney (377a). The manner by which the epididymal epithelium forms an organic solute–rich environment is not completely known, but there is considerable evidence to suggest that at least several transporters are involved in moving the solute from the blood into the lumen; in addition, the epididymis synthesizes and secretes solutes into the lumen. For example, L-carnitine is transported into the epididymal cells and lumen, reaching concentrations as high as 50 to 60 mM in some species (378–382). The high intraluminal concentration of L-carnitine translates into a remarkable 2,000-fold gradient of L-carnitine across the epididymal epithelium. Studies suggest that the distal caput, corpus, and proximal cauda epididymidis are the regions involved in the active transport of L-carnitine (383–388). The mechanism of L-carnitine transport in the epididymis is unknown, but has been suggested to involve an active transport system (383,384,386,389) that is androgen dependent (378,380,390,391).

More recent studies have suggested that L-carnitine is transported from blood into cells by the organic cation/carnitine transporter OCTN2 (392), and then presumably from cells into the lumen by the carnitine transporter CT2, which has been identified in the human epididymis (393). Several transporters, such as OCTN2 and SMIT (a sodium–myoinositol transporter), have TonE binding motifs on their promoters that bind a transcription factor, TonE binding protein (TonEBP; Rodriguez and Hinton, unpublished observations), that is expressed during periods of hypertonicity; these data suggest that the organic solutes play a role in the regulation of water movement across the epididymal cell membranes. This is particularly important because in the epididymis of most species, spermatozoa are exposed to a hyperosmotic environment. Disruption of the ability of spermatozoa to regulate their cell volume has been suggested to cause male infertility [reviewed in Cooper et al. (394)].

The high levels of organic solutes measured in the cauda epididymal fluid of several species suggest that these solutes play a similar role in the long-term storage of spermatozoa in this region. Likewise, in bats that store spermatozoa in the cauda epididymidis for up to 6 months, the spermatozoa are stored in a supraphysiologically high osmotic environment with a luminal fluid osmolality measured at greater than 1,000 mOsm/kg water (395); the mechanisms for generating and maintaining such a high osmolality are unknown. Taurine and glutathione are both found in epididymal luminal fluid (396,397), and because both of these compounds have been implicated in protecting cells from oxidative stress, it would not be surprising if they have a similar role in the epididymis.

The transport of sugars and amino acids, including inositol, 3-O-methyl-D-glucose and 2-deoxy-D-glucose (nonmetabolizable forms of glucose), and α-aminoisobutyric acid (AIB; a nonmetabolizable neutral amino acid), has been studied in the rat epididymis (398–403), yielding several observations. First, glucose entered by a facilitated diffusion mechanism, and this uptake appeared to be similar across all epididymal regions. Second, glucose transport was inhibited by the male infertility agents α-chlorhydrin, 5-thio-D-glucose, and 6-chloro-6-deoxy-glucose. Third, transport of inositol and AIB was highest in the initial segment compared with the rest of the epididymis. Finally, transport of inositol and AIB was saturable and, presumably, transport of inositol was through an active mechanism, whereas that of AIB was through a facilitated diffusion mechanism. Although the GLUT family of glucose transporters has been well studied in numerous tissues, only GLUT8 has been identified in epididymal spermatozoa (404) and tissue (43). Presumably, the glucose and amino acid needs of the epididymis reflect the metabolic needs of

the epididymis and maturing spermatozoa. However, more studies are needed to examine the roles played by each solute. The role of known specific ion and organic solute transporters that have been identified in the epididymis is discussed in greater detail later in this chapter.

The androgen luminal fluid microenvironment has received little attention recently, but past studies revealed that the spermatozoa are exposed to a rich and complex androgen environment as they progress along the duct [reviewed in Turner (359)]. In several species, including rat, human, hamster, stallion, and bull, the epididymal luminal fluid concentrations of testosterone and dihydrotestosterone (DHT) exceed the blood concentrations (405–407), with DHT concentration being very high in the caput region. The high luminal fluid androgen concentrations are maintained by androgen-binding protein (ABP) because studies by Turner et al. (1984) (407) showed that intraluminal ABP concentrations are equimolar with total androgen levels. It has been proposed that the ABP–androgen complex plays a role in epididymal function (408), but confirmatory experiments have yet to be designed to test this hypothesis.

Protein Microenvironment

The protein composition of epididymal luminal fluid has been well studied for a number of species [reviewed in Dacheux et al. (93,409)]. Spermatozoa are exposed to varying protein concentrations as they move along the seminiferous tubule (approximately 6 to 10 μg/μL), to the rete testis (approximately 1 μg/μL), to the caput and cauda epididymidis (approximately 25 to 30 μg/μL). Because water is being absorbed along the epididymal duct, a higher protein concentration would have been expected in the cauda compared with the caput epididymidis. To account for this difference, proteins are presumably being absorbed or degraded, or are being utilized by the spermatozoa. However, even though the net movement of protein is by absorption, many proteins are still being synthesized and secreted into the duct.

The proteins in epididymal luminal fluid have been analyzed by one- and two-dimensional gel electrophoresis (410), generating characteristic patterns of protein profiles for several species; many of the proteins have now been identified using mass spectrophotometric methods. Using an in vivo split-drop, stopped-flow microperfusion technique together with two-dimensional gel electrophoresis and both silver staining and Western blot analyses, Hinton and colleagues (411,412) showed in vivo protein secretion into the lumen of discrete epididymal segments and regions. Later studies by Turner and colleagues (413,414) obtained similar results when the interstitium was perfused with radiolabeled methionine and luminal fluid collected by micropuncture and analyzed for radiolabeled proteins.

Epididymal luminal fluid contains a large repertoire of proteins, including enzymes, growth factors, lipid-binding proteins, iron-binding proteins, proteins that may be involved in protection or in sperm–egg binding, and other proteins such as the lipocalins, clusterin, lactoferrin, and the cholesterol transport protein CTP/HE1 [reviewed in Dacheux and Dacheux (93)]. Many of these families of proteins are discussed in later sections.

REGULATION OF EPIDIDYMAL FUNCTIONS

Hormones

In 1926, pioneering studies by Benoit demonstrated that the epididymis depends on an unknown testicular substance for maintenance of its structure and functions; he showed that the ratio of nucleus to cytoplasm in the mouse epididymis decreased after birth as the epididymis differentiated, and increased dramatically after orchidectomy, as the epididymis dedifferentiated (12). This regulatory substance was identified 5 years later as testosterone (415). Since then, over a thousand papers have appeared on the response of the epididymis in numerous mammals to androgen withdrawal and replacement on a wide variety of end points, ranging from morphological to biochemical and molecular.

The main approach to understanding the effects of androgen withdrawal on the epididymis has been removal of the testis. It is clear that this approach causes loss not only of androgens but of estrogens and any other testicular factor that may affect the epididymis.

Orchidectomy causes a decrease in epididymal weight that is less marked than that of sex accessory tissues such as the prostate or seminal vesicles (416,417). Unlike the case with other androgen-dependent male reproductive tissues, testosterone replacement, even at supraphysiological levels, only partially restores epididymal weight; this is presumably due to the large proportion (nearly half) of epididymal weight that is attributable to spermatozoa and the luminal fluid bathing them (417,418). In the androgen-deprived state, spermatozoa become immotile, lose the ability to fertilize, and die (12,161,419). After orchidectomy, the luminal diameter and epithelial cell height decrease and the intertubular stroma increases (420). The smooth endoplasmic reticulum content is dramatically reduced, whereas the extent of decline in the Golgi

apparatus is less pronounced (421–423). Morphological changes in principal cells suggest that these cells are particularly sensitive to androgen levels, in contrast to the other epithelial cell types, which appear to be less affected by orchidectomy (101). The secretory function of principal cells becomes compromised in the androgen-deprived state. In addition to the virtual disappearance of endoplasmic reticulum from their apical cytoplasm, principal cells undergo a striking loss of apical microvilli from their surface, as well as lysosome accumulation, vacuolization, disappearance of vesicles from the cell apex, and increased endocytosis (101,287). Epididymal androgen receptors and 5α-reductase activity are both decreased in the androgen-deficient state (417,424–426), suggesting that the mechanisms of androgen action are compromised by androgen withdrawal. Total epididymal protein, RNA, and DNA content are reduced after orchidectomy, but the DNA concentration is apparently increased (427). The increase in DNA concentration is attributed to the concomitant decline in cell volume; this is thought to be the principal mechanism by which the epididymal epithelium regresses after orchidectomy.

Restoration of circulating testosterone levels appears sufficient to reverse regressive changes in the caput, corpus, and cauda epididymidis after orchidectomy but not in the initial segment, even when supraphysiological doses of testosterone are administered (417,423,428–432). At 3 days after orchidectomy with testosterone or DHT replacement to circulating levels, a time at which the prostate shows maximum rates of DNA synthesis, the epididymal labeling index, measured by [^{3}H]-thymidine incorporation and mitotic index, is low except in the corpus region (433). The lack of effect of androgens on mitotic rate in the epididymis of the adult animal distinguishes this tissue from other androgen-dependent tissues such as the prostate and seminal vesicles. This characteristic of the epididymal epithelium suggests that it may contain antiproliferative signals that inhibit cellular proliferative capacity in response to androgen stimulation or other stimuli. Interestingly, B-myc, a transcription factor known to inhibit cellular proliferation, is highly expressed in the epididymal epithelium (434).

Withdrawal of androgen stimulation by orchidectomy induces a wave of apoptotic cell death in the epididymis, beginning in the initial segment and moving over several days to the cauda epididymidis (435,436). Apoptosis in the initial segment seems to be caused by withdrawal of androgens and luminal components from the testis, and appears to be p53 independent. Using the entire epididymis, Bcl-2, an antiapoptotic factor (437), was found to be suppressed by 36 hours after orchidectomy; this was followed by the appearance of Fas and DNA fragmentation in the epididymal epithelium at 48 hours postorchidectomy (438). Mutant mice null for Fas (*lpr*) showed no epididymal regression or DNA fragmentation after orchidectomy. These data suggest that the regression of the epididymal epithelium after orchidectomy may be regulated through the Fas pathway (438).

In addition to androgens, many other hormonal factors have been postulated to play a role in regulating epididymal function; these are listed in Table 4 (supplemental material on web site). Of special note, however, is estradiol. It has been known for many years that the administration of estrogens could affect the male reproductive system in general (439–448) and the epididymis in particular (166,449). With the advent of aromatase-specific inhibitors and null mutant mice for the two isoforms of the estrogen receptor (discussed later), it is likely that estrogens will be shown to act as regulators of some specific epididymal functions; however, much remains to be done to resolve exactly what role this steroid family plays.

Testicular Factors

In addition to depending on the presence of circulating androgens, the epididymis also depends on the presence of luminal fluid factors originating from the testis or the epididymis itself. Without testicular luminal fluid factors, many cells in the initial segment undergo apoptosis within 24 hours (435,436,450). Therefore, it is not surprising that ligating the efferent ducts results in changes in morphology and gene expression in the initial segment [reviewed in Robaire and Hermo (1), Cornwall et al. (91), and Hinton et al. (451,452)]. From these studies, it has been postulated that a factor or factors originating from the testis is responsible for maintaining the integrity and survival of cells in the initial segment. This type of paracrine secretion has been termed *lumicrine* because this mode of regulation occurs in a duct/tubal system (452). A general scheme depicting how factors secreted directly into the epididymis through the efferent ducts can affect activities of cells in the initial segment and how such effects can cascade down the duct is shown in Fig. 12. Perhaps the first study demonstrating lumicrine regulation in the male excurrent duct system was that by Skinner and Rowson (1967; 1968) (453,454). These investigators showed that the ampulla of the vas deferens weighed less, contained less fructose, and was smaller in diameter after unilateral vasectomy compared with the ampulla on the intact control side. Skinner and Rowson (454) suggested that vasectomy

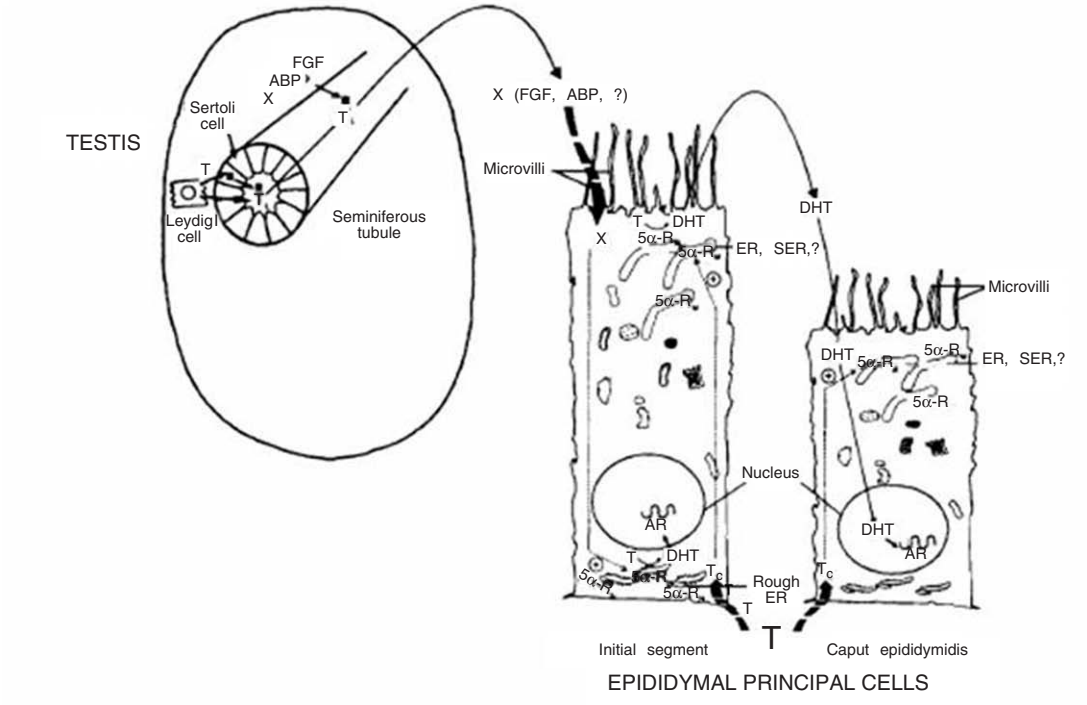

FIG. 12. Proposed mechanism for the regulation of 4-ene steroid-5α-reductase and androgen action in the rat epididymis. Leydig cells synthesize testosterone (T), which stimulates the Sertoli cell to secrete androgen-binding protein (ABP) and other proteins into the lumen of the seminiferous tubule. In the initial segment of the epididymis, the paracrine/lumicrine regulator (e.g., fibroblast growth factor [FGF]), ABP is proposed to be the paracrine factor regulating the infranuclear localized 5α-reductase enzyme. In contrast, the apically localized 5α-reductase enzyme (microsomal 5α-reductase) found throughout the epididymis is under the control of circulating androgens. The synthesis of locally high concentrations of dihydrotestosterone (DHT) in the initial segment of the epididymis provides an excellent mechanism for stimulating critically important androgen-dependent genes in this region of the epididymis and for providing the most active androgen to mediate androgen action in the rest of the tissue. 5ur-RN, nuclear 5α-reductase; 5-RM, microsomal 5α-reductase; ER, endoplasmic reticulum; SER, smooth endoplasmic reticulum. (Modified from Robaire, B., and Viger, R. S. [1995]. Regulation of epididymal epithelial functions. *Biol. Reprod.* 52, 226–236.)

prevented luminal fluid testosterone from reaching the ampulla, and perfused the vas deferens of an orchidectomized ram with testosterone to test this hypothesis. Their data show that the ampulla of the perfused side weighed more and contained more fructose and citric acid compared with the nonperfused control side. Similarly, studies by Abe et al. (1984) (48) show that ligation of the mouse corpus epididymidis results in abnormal differentiation of the principal cells downstream from the ligature. Hence, lumicrine regulation may occur not only between the testis and epididymis but between regions of the epididymis, including the vas deferens.

The cells in zones 1a, 1b, and 1c [see Reid and Cleland (57) for zones] of the rat initial segment have been shown to be the cells most highly regulated by testicular luminal fluid factors. Whether there are more distal effects along the epididymis and the extent to which this regulation occurs in other species need further investigation. Although testicular luminal fluid steroids, androgens and estrogens, are the most obvious candidates for regulating initial segment function, several lines of evidence suggest that additional factors are important. Using a histological approach, Fawcett and Hoffer (1979) (423), supported the idea that factors other than androgens are responsible for the maintenance of initial segment function because the morphology of the initial segment cells failed to return to normal after efferent duct ligation and testosterone treatment. Likewise, Nicander and colleagues (1983) (450), suggested that because the cells in the initial segment are highly mitotic, they are more dependent on the presence of a mitogen in testicular luminal fluid.

The first nonsteroidal molecule proposed to be a testicular factor acting on the epididymis was ABP (408,455). This hypothesis stemmed from a series of studies demonstrating that the steroid 5α-reductase activity in the initial segment of the epididymis fell after efferent duct ligation or unilateral orchidectomy;

it could not be maintained by administration of testosterone, even at very high doses. Luminal fluid ABP was also proposed as the factor that enhances the function of ovine epididymal principal cells in culture (456), and appears to play a role in protein synthesis in the caput region (457). However, although γ-glutamyl transpeptidase IV mRNA is also controlled in a lumicrine manner in the initial segment of the epididymis, ABP does not play a role in its expression (458). Instead, FGFs may be partly responsible for regulation of the expression of this gene (458,459). It has been postulated that luminal fluid growth factors interact with their cognate receptors on the apical cell surface of initial segment cells, whereupon a signal transduction pathway is initiated (e.g., mitogen-activated protein kinase [MAPK], phosphatidylinositol-3 kinase); transcription factors are then activated, presumably by phosphorylation, leading to transcription of target genes (452,459). In support of this hypothesis, soluble FGFs (2, 4, and 8) have been identified in testicular luminal fluid (458,459), and FGF receptor (FGFR)-1 IIIc (both long and short forms) has been identified in the principal cells in the initial segment. Further, the activity of the MAPK pathway and the expression of members of the polyomavirus enhancer activator 3 (PEA3) transcription factor family (PEA3, ERM, ER81) are decreased after loss of testicular factors [(460); J. L. Kirby, B. V. Troan, and B. T. Hinton, unpublished observations]. Interestingly, several genes, including steroid 5α-reductase (461), expressed in the rat initial segment contain multiple PEA3 family member–binding motifs, and most of these genes are regulated by testicular luminal factors. In addition, neurotrophins (nerve growth factor, NT-3) and their cognate receptors, Trks and p75, may also play a role in the regulation of initial segment function (S. Crenshaw and B. T. Hinton, unpublished observations). Crucial to the coordinated functions of the FGFR and Trk receptor pathways may be adaptor proteins such as FRS2 and Shc (S. Crenshaw and B. T. Hinton, unpublished observation).

Another "factor" that has been postulated to regulate initial segment function is the presence of spermatozoa (462). At first, cells seem to be an unusual "ligand," yet, on closer inspection, there may be a sound basis for this hypothesis. Spermatozoa themselves may not be the ligand, but it is possible that the ligands they carry and transport to the different epididymal regions are important; many molecules are known to bind to spermatozoa and potentially shed in the epididymis. Interestingly, testicular spermatozoa stain positively for growth factor receptors (463); it is tempting to speculate that on entering the initial segment, growth factors dissociate from the sperm surface and become available to stimulate their cognate receptors on the epididymal apical cell surface.

If it is assumed that the lumicrine mode of regulation occurs throughout the epididymis, there are several potential key points that emerge regarding the regulation of the initial segment, and possibly the entire epididymis. First, the cells in the adult initial segment are being constantly stimulated by FGFs, yet the cells are not proliferating; likewise, the MAPK pathway is constitutively switched on in these cells. However, not all cells in the initial segment show MAPK pathway activity (J. L. Kirby et al., unpublished observation). Hence, this raises a second point that cells may be in communication with each other such that they cycle between "on" and "off" states with respect to the response to growth factors. This may explain the characteristic checkerboard pattern seen when the tissue is stained for many different proteins; it would enable the initial segment to function normally as a whole, yet with each cell autonomous with respect to the regulation of its repertoire of target genes but susceptible to influence by the state of its neighboring cells. This theory may also explain why cells do not proliferate in the initial segment after constant stimulation with growth factors. At any time, there is sufficient stimulation of growth factors to upregulate target genes, yet, because of complex negative feedback systems (e.g., involving the brain-derived neurotrophic factor/NT-3–Trk receptors and adaptor proteins), those cells do not enter the cell cycle pathway. However, the cells need to be responsive to growth factors fairly rapidly after the "off" state because lack of stimulation by testicular factors leads to apoptosis (S. Crenshaw and B. T. Hinton, unpublished observations). Future experiments will be needed to test this hypothesis, but it will also be interesting to know whether the recycling hypothesis for many of the genes applies in all the cells throughout the epididymis. This hypothesis may also have implications with respect to the low rate of primary or secondary cancers observed in the epididymis.

MAJOR PROTEIN FAMILIES IN THE EPIDIDYMIS AND THEIR REGULATION

General Principles

The division of the epididymis into segments has more than anatomical implications. For many years, the differential expression of proteins and mRNA along the epididymis has become a hallmark of this tissue. Indeed, the large number of gene expression profiling studies (311,353,464–468a), proteomic analyses [reviewed in Dacheux and Dacheux (93), Gatti et al. (469), Chaurand et al. (470), and Umar et al. (471)],

and immunohistochemical studies [e.g., (107,109, 472–474)] have all revealed that nearly all molecules have characteristic longitudinal expression profiles in the epididymis of all species examined. Data on the gene expression profiles of the different segments of rat and mouse epididymis are available at the website for this article (www.ttuhsc.edu/cbb/faculty/cornwall/default.asp), and at www.wsu.edu/%7Egriswold/microarray/epididymis_dht/. In addition, tables of genes encoding distinct classes of proteins (enzymes, signaling, DNA binding) that exhibit regionalized expression in the epididymis are provided in the supplemental material on this chapter's web site. Table 2 lists genes encoding secretory proteins that may play direct or indirect roles in the sperm maturation process, whereas Table 3 provides a list of genes that encode cellular proteins that likely perform regulatory functions.

Several comprehensive reviews describing the longitudinal profiles of both proteins and mRNA have been published recently. Although all of these studies clearly demonstrate that there is a highly regionalized activation and deactivation of gene expression, the underlying mechanisms that allow for this regulation are unclear. Regionalized expression of transcription factors (see later) is beginning to provide some insight into this question, but the relationship between the luminal, endocrine, and cellular components that allow for such highly regionalized expression of genes and proteins will require many more years to resolve.

Perhaps as challenging to understand is the checkerboard pattern of expression observed in cross sections of individual tubules. This characteristic pattern is usually seen when a given protein or mRNA is first expressed along the duct or when the expression terminates. It must be assumed that adjacent principal cells either use different sensing mechanisms for the triggering signal, or more likely function as a syncytium to optimize coordinately the degree of expression of a given gene or protein. Examples of this checkerboard pattern of expression are shown in Fig. 13.

As might be expected based on their appearance and proposed functions, the different cells of the epididymal epithelium express different genes and proteins.

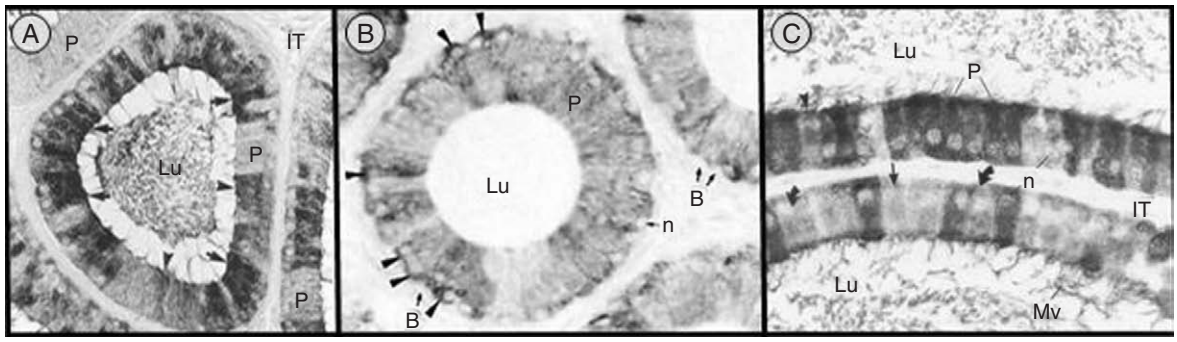

FIG. 13. Representative examples of the checkerboard pattern of expression seen immunohistochemically along the epididymis. **A:** High-power light micrograph of tubules of the distal area of the initial segment immunostained with anti-immobilin. Although many principal cells are reactive, they show a variable staining pattern. Several principal cells are intensely stained throughout their cytoplasm (*arrows*), whereas others are moderately or weakly stained (*arrowheads*); a few are unreactive (P). The luminal content (Lu) is well stained. IT, intertubular space. (Magnification ×350; modified from Hermo, L., Oko, R., and Robaire, B. [1992]. Epithelial cells of the epididymis show regional variations with respect to the secretion or endocytosis of immobilin as revealed by light and electron microscope immunocytochemistry. *Anat. Rec.* 232, 202–220.) **B:** High-power light micrographs of the proximal initial segment of the epididymis. Epithelial principal cells (P), nuclei of principal cells (n), basal cells (B), and lumen of the duct (Lu) are shown. Intense staining in the infranuclear region of the principal cells is designated by the *arrowheads*. Immunostaining reaction is confined to an oval region present above the nuclei of the principal cells (*large arrows*). (Magnification ×400; modified from Robaire, B., and Viger, R. S. [1995]. Regulation of epididymal epithelial functions. *Biol. Reprod.* 52, 226–236.) **C:** Higher-power micrograph of portions of adjacent tubules of the caput epididymis immunostained with anti–SGP-2. Note intense immunoperoxidase staining reaction product over some epithelial principal cells (P). Other principal cells are moderately (*arrowheads*) or weakly (*curved arrows*) immunoreactive. A few principal cells are virtually unreactive (*arrow*). Note that the reaction product extends throughout the entire cell cytoplasm (i.e., basal, supranuclear, and apical regions), leaving only the nucleus (n) unstained. The microvillar (Mv) border of the principal cells and sperm in the lumen (Lu) of the tubules are reactive. IT, intertubular space. (Magnification ×600; modified from Hermo, L., Wright, J., Oko, R., and Morales, C. R. [1991]. Role of epithelial cells of the male excurrent duct system of the rat in the endocytosis or secretion of sulfated glycoprotein-2 (clusterin). *Biol. Reprod.* 44, 1113–1131.)

Isolating each of the different cell types has been a challenge, but with the development of tools such as laser capture microscopy, it should become possible to resolve the profiles of genes and proteins expressed in each type of epithelial cell. Using immunohistochemistry, however, it has been possible to demonstrate the highly specific expression of certain proteins in principal, basal, clear, narrow, and halo cells. Figure 14 presents examples of the selective marking of each of these cell types. To provide potential tools to help identify cell types in vivo and in vitro, representative proteins expressed selectively in individual cell types of different regions of the epididymis are listed in Table 4 (supplemental material on web site); some of these proteins are secreted, whereas others are retained in the cell.

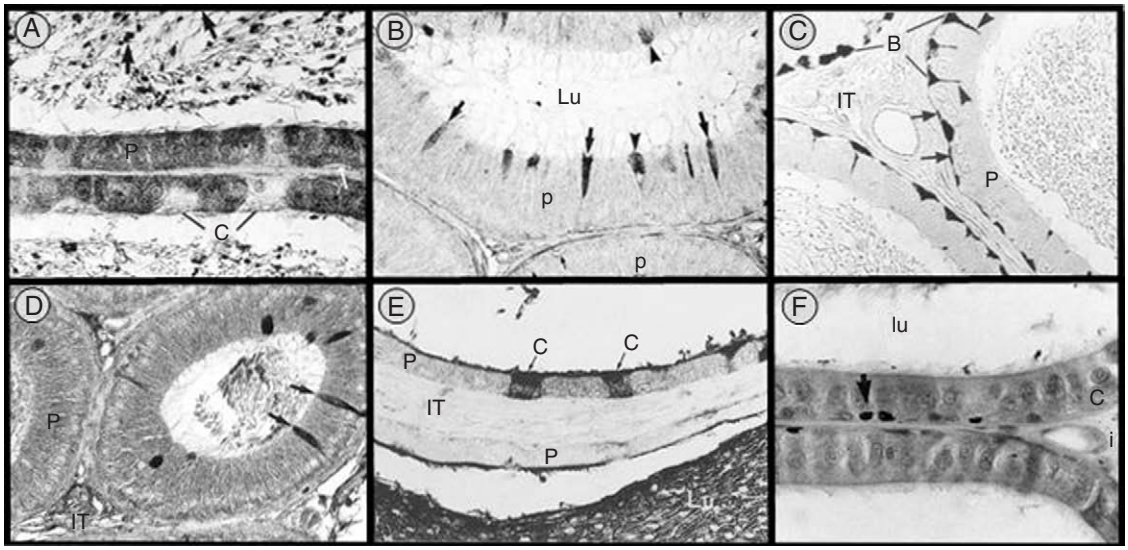

FIG. 14. Cell-specific expression of proteins along the rat epididymis. Examples of immunolocalization of proteins in each of the major cell types are presented. **A:** Principal cells (junction of the caput–corpus of the epididymis) are immunostained for the glutathione S-transferase (GST) Yo subunit. The epithelial principal cells (P) are well stained, but basal (*white arrow*) and clear (C) cells are unreactive. Cytoplasmic droplets of spermatozoa (*arrows*) in the lumen are intensely reactive. (Magnification ×365; modified from Veri, J. P., Hermo, L., and Robaire, B. [1994]. Immunocytochemical localization of glutathione S-transferase Yo subunit in the rat testis and epididymis. *J. Androl.* 15, 415–434.) **B:** Apical cells (*arrowheads*) and narrow cells (*large arrows*) of the proximal initial segment of the epididymis are intensely reactive when immunostained with anti-Yo GST antibody, whereas principal cells (P) and basal cells (*small arrows*) are unreactive. Lu, lumen. (Magnification ×320; modified from Adamali, H. I., and Hermo, L. [1996]. Apical and narrow cells are distinct cell types differing in their structure, distribution, and functions in the adult rat epididymis. *J. Androl*. 17, 208–222.) **C:** Basal cells. The only cell type of the epithelium of the corpus epididymidis that is intensely reactive is the basal cell (B) when immunostained with antibodies to the Yf (Yp) subunit of GST. These cells often show slender processes (*arrows*) extending from the expanded main cell body along the basement membrane and, occasionally, toward the lumen (*arrowheads*). Principal cells (P) appear unreactive. Spermatozoa in the lumen are unreactive. IT, intertubular space. (Magnification ×440; modified from Veri, J. P., Hermo, L., and Robaire, B. [1993]. Immunocytochemical localization of the Yf subunit of glutathione S-transferase P shows regional variation in the staining of epithelial cells of the testis, efferent ducts, and epididymis of the male rat. *J. Androl*. 14, 23–44.) **D:** Narrow cells. Initial segment of epididymides immunostained with anti–carbonic anhydrase II antibody. Intense reaction is localized over narrow cells. P, principal cell; IT, intertubular space; *arrows*, narrow cells. (Magnification ×350; modified from Hermo, L., Adamali, H. I., and Andonian, S. [2000]. Immunolocalization of carbonic anhydrase (CA) II and H^+ V-ATPase in epithelial cells of the mouse and rat epididymis. *J. Androl*. 21, 376–391.) **E:** Clear cells. Tubules of the distal area of the cauda epididymidis immunostained with anti-immobilin. Note that several epithelial clear cells (C) are intensely reactive throughout their cytoplasm, whereas other cells remain unreactive. IT, intertubular space; P, principal cells. (Magnification ×375; modified from Hermo, L., Oko, R., and Robaire, B. [1992]. Epithelial cells of the epididymis show regional variations with respect to the secretion or endocytosis of immobilin as revealed by light and electron microscope immunocytochemistry. *Anat. Rec*. 232, 202–220.) **F:** Halo cells. Immunostaining with an antibody for helper T lymphocytes (CD4) in the cauda epididymidis. *Arrows* indicate CD4+ cells. lu, lumen. (Magnification ×450; modified from Serre, V., and Robaire, B. [1999]. The distribution of immune cells in the epithelium of the epididymis of the aging brown Norway rat is segment-specific and related to the luminal content. *Biol. Reprod.* 61, 705–714.)

Functional Families

Receptors and Their Ligands

Androgen Receptor, Androgen-binding Protein, and Steroid 5α-Reductases. The limiting factor in determining the action of testosterone on cells is not clear. The presence of both the androgen receptor and an agonist are obviously necessary, but there are several lines of evidence that indicate that it is not primarily the absolute number of androgen receptors present but rather the amount of the ligand that is limiting. In addition, testosterone, a highly lipophilic molecule, is carried to sites distant from where it is made by binding proteins such as ABP. Testosterone can act directly on the androgen receptor in some tissues, such as muscle; in other tissues, such as some brain nuclei, testosterone needs to be aromatized to estradiol to mediate its action through the estrogen receptor; whereas in still other tissues, it is converted to a 5α-reduced metabolite, DHT. DHT binds to the androgen receptor with higher affinity than testosterone [reviewed in Blanchard and Robaire (475)]. The latter mode operates in many androgen-dependent tissues such as the epididymis, prostate, seminal vesicles, and skin. The rate-limiting enzyme in the pathway leading from testosterone to its 5α-reduced metabolites is steroid 5α-reductase (5α-R, EC 1.3.1.22).

Androgen Receptor. The first report that exogenously administered testosterone was converted to DHT and bound to a cytosolic protein in the epididymis (the androgen receptor) (476) was nearly coincident with the discovery of a second androgen-binding component (ABP) in testis and epididymal cytoplasmic fractions (476a). Although both of these proteins bind androgen with high affinity, they are distinct molecules. For example, although both of these proteins have high affinity for DHT, the androgen receptor has a slow dissociation rate for DHT ($t_{1/2}$ 0°C > 4 days), whereas ABP dissociates at a much faster rate ($t_{1/2}$ 0°C = 6 minutes). Androgen receptors have been found in the epididymis of all species examined to date (rat, rabbit, dog, ram, monkey, and human) (476,476b–476h). In other mammalian tissues, a single gene for the androgen receptor has been identified. In humans, the gene spans approximately 90 kilobases, and it consists of 8 exons on chromosome Xq11-12. The coded protein is approximately 110 kDa, and multiple transcripts are produced with variable lengths of a polymorphic region of CAG repeats in exon 1 (477,478). Recently, a variant of the human androgen receptor (AR45), lacking the entire region encoded by exon 1 of the gene but retaining the DNA-binding and steroid-binding domains, has been identified in heart and muscle tissues (479). The potential presence of further variants in other tissues may help explain the tissue specificity of androgen action.

There are relatively small changes in the concentration of mRNA for the androgen receptor along the epididymis (480,481). When changes are noted, they often reflect a decline in the concentration of androgen receptors in the progression toward the distal end of the epididymis. For example, in humans, both the mRNA protein for the androgen receptor are found throughout the tissue, with high levels in the distal caput and proximal corpus and declining in the caudal region, including the vas deferens. Interestingly the efferent ducts do not show any immunostaining, whereas the vas deferens is only weakly positive (476d). An exception is the ram, where androgen receptor concentrations are similar in the caput and cauda epididymidis but are less than 25% of these levels in the corpus epididymidis (482). In both the goat and the rat, epithelial cells stain fairly evenly for androgen receptors throughout the duct (483,484).

It is clear that principal cells stain heavily for androgen receptors, but the relative concentrations of this receptor in basal, clear, narrow, and apical cells are not known. However, the immunolocalization of androgen receptors in all epididymal epithelial cells has been reported for monkey, mouse, and goat (476h,485,486), whereas in the rat, it is apparent that clear cells do not stain for the androgen receptor (426). Because of their origin, and as one might anticipate, halo cells do not stain for the androgen receptor in any species examined. Peritubular cells occasionally display weak signals, whereas interstitial cells or blood vessels are consistently negative. When noted, the intracellular localization of the androgen receptor is distinctly nuclear.

Very few studies have focused directly on the mechanism of action of the androgen receptor in the epididymis; however, with the advent of several stable cell lines from this tissue (487–489), such studies are likely to be undertaken in the near future. The characteristics and mode of action of the androgen receptor as a transcription factor (490,491) in the epididymis seem to resemble those in other tissues. However, androgen signaling, although essential, is not sufficient for epididymis-specific gene expression because the androgen receptor is expressed in many tissues. One mechanism proposed to explain the specificity of transcriptional responses to androgen is the tissue-specific combination of transcription factors binding to the promoter region of androgen-regulated genes (492). For example, the epididymal and prostatic lipocalin genes for epididymal retinoic acid–binding protein (E-RABP) and probasin are regulated by the androgen receptor. In the prostate, the transcription factor Foxa1 (forkhead box A1) is

required for probasin gene expression (493). In the epididymis, Foxa2, but not Foxa1, is expressed, interacts with the androgen receptor, and binds to the promoter of the E-RABP gene. Interestingly, overexpression of Foxa2 suppresses androgen activation of the E-RABP promoter, but increases activation of the prostate-specific promoter in an androgen-independent manner (494). Because Foxa1 and Foxa2 expression is restricted to the prostate and epididymis, respectively, it suggests that these transcription factors have distinct and different action on androgen receptor–regulated genes in different male reproductive organs. It is tempting to speculate that these differential responses play a role in the different responses of these two organs to oncogenic transformation.

Little is known about the regulation of expression of the androgen receptor in the epididymis. In the goat, circulating androgens seem to be sufficient to regulate the expression of both androgen and estrogen receptors (486). In the rat, androgen withdrawal by the administration of a luteinizing hormone–releasing hormone antagonist results in a more extensive and rapid decline in androgen receptors in stromal than in epithelial cells; androgen replacement results in a complete restoration of androgen receptors, indicating that stromal cells are more sensitive than epithelial cells to the regulation of androgen receptor concentrations by androgen (426).

Androgen-binding Protein and Its Receptor. ABP is one of the first proteins shown to be synthesized by Sertoli cells of the testis (495) and has been found in several species, including the rat (476a), rabbit (496), guinea pig (497), ram (476b), monkey (498), and human (499). It is secreted in both the luminal and basal compartments of the seminiferous tubules, but the overwhelming majority, more than 80%, is directed toward the epididymis (500). Plasma sex hormone–binding globulin (SHBG) shares the same amino acid sequence with ABP; these molecules differ only in the types of oligosaccharides associated with them (501). The biological significance of these differences is not clear in light of the fact that SHBG and a glycosylated mutant have similar binding characteristics to sex steroids (502). ABP concentrations are highest in the caput and lowest in the cauda epididymal segments of most species examined, including primates; this distribution correlates well with DHT content (85,503,504). ABP is synthesized and secreted by epididymal principal cells all along the epididymis (505), but in several species it is also taken up into the epididymal principal cells of the initial segment and caput epididymidis (495,506,507) through a receptor-mediated process (505,508–511). Testicular ABP production is regulated by androgens (512), whereas that of the epididymis seems to be under the control of a testicular factor (505).

Several functions have been ascribed to ABP. It has been proposed to act as a carrier of androgens to the epididymis (513). In the principal cells, testosterone is released from ABP and converted to the more potent androgen, DHT, by the enzyme 5α-reductase. ABP has high affinity for 5α-DHT ($K_a = 1.25 \times 10^9$ M^{-1}) and testosterone ($K_a = 0.5 \times 10^9$ M^{-1}); rapid dissociation of testosterone from ABP (6 minutes) is consistent with a role for ABP as a carrier molecule for testosterone. In addition to its role as a carrier molecule, ABP has been suggested to be an androgen sink in the seminiferous tubules of the testis (513) because the concentration of androgens in the seminiferous tubules needs to be higher than that in the peripheral circulation (514–516) to maintain spermatogenesis. ABP has also been proposed to regulate steroid 5α-reductase type 1 (408,456,517).

To demonstrate unequivocally a function for ABP in spermatogenesis or in the epididymis would require the development of a null mutation model; this has not yet been accomplished. However, mice overexpressing ABP (518) show fertility defects stemming from spermatogenic dysfunction in the testis, which results in reduced numbers of spermatozoa in the cauda epididymidis. Although in these transgenic lines, ABP levels are increased in the epididymis, the consequence of this for epididymal function is not evident.

Although it is clear that testicular ABP enters the principal cells of the proximal segments of the epididymis, the mechanism of internalization is still not fully understood. The presence of high-affinity binding sites ($K_{ass} = 3.7$ nM^{-1}; 4.5×10^{11} sites/mg protein) on the apical surface of epididymal principal cells and the demonstration that the number of these receptors is fivefold lower in the cauda than in the caput of the epididymis have led to the proposal that ABP internalization is a receptor-mediated process in both rats and monkeys (504,509,510).

Steroid 5α-Reductases. The selective presence of radioactive DHT in epididymal cell nuclei after injection of radiolabeled testosterone (519), and the demonstration using micropuncture studies that, beyond the efferent ducts, the predominant androgens in epididymal fluid are 5α-reduced metabolites of testosterone [reviewed in Turner (85)], are two of the key findings establishing the central role of DHT, and hence steroid 5α-reductase, in mediating androgen action in the epididymis. The discovery that testosterone is rapidly converted by the epididymis to 5α-reduced products was first made almost simultaneously by two different groups (520,521). The epididymis is one of the tissues with the highest level of activity anywhere in the body (521).

In the mouse, rat, dog, monkey, and human, two 5α-reductases (isozymes), with different tissue

distributions, have been identified. Although several theories have been put forward regarding the relative role of these two isozymes (522,523), there are few solid data to allow resolution of their relative roles. The consequences on the female of a null mutation in 5α-reductase 1 have been described (524). Null mutations for these two genes are compatible with male fertility; however, no detailed studies on spermatozoa or the epididymis of mice carrying null mutations for these genes have yet appeared. To understand the regulation of this enzyme activity in the epididymis, it is necessary to determine where and how the proteins are expressed, as well as the translation and, ultimately, the factors regulating transcription of the genes for this enzymatic activity.

Enzyme activity is expressed in a striking positional gradient as well as in different subcellular fractions in the adult rat epididymis (455,525). The activity associated with the nuclear fraction is highest in the initial segment, where it is higher than in any other male reproductive tissue, and declines dramatically in more distal segments of the tissue. The hormonal regulation of epididymal 5α-reductase activity is complex [reviewed in Robaire and Viger (517) and Ezer and Robaire (526)]. This was the first enzyme shown to be regulated by testicular factors as well as by circulating testosterone, depending on which segment and subcellular fractions were examined. Whereas nuclear fraction epididymal 5α-reductase activity is regulated in a paracrine or lumicrine manner by a substance directly entering the epididymis through the efferent ducts, microsomal 5α-reductase activity is found throughout the epididymis but is present at a lower level and is regulated by circulating androgens.

The two mRNAs for 5α-reductase types 1 and 2 are the products of separate genes (527,528). The type 1 mRNA isozyme is most abundantly expressed in the initial segment of the epididymis, with a positional gradient that is similar to the enzyme activity. Studies of the endocrine and developmental regulation of the 5α-reductase type 1 mRNA show that (a) orchidectomy results in a decrease in type 1 mRNA levels in all epididymal segments; (b) after orchidectomy, high-dose exogenous testosterone maintains 5α-reductase type 1 mRNA levels at control levels in all regions of the epididymis except the initial segment; (c) unilateral orchidectomy and efferent duct ligation cause a dramatic decrease in type 1 5α-reductase, selectively in the initial segment of the epididymis; and (d) only the type 1 transcript is developmentally regulated. Therefore, with regard to enzyme activity, the primary regulator of 5α-reductase type 1 mRNA expression in the initial segment is a paracrine/lumicrine factor of testicular origin [reviewed in Robaire and Viger (517) and Ezer and Robaire (526)].

The 5α-reductase type 2 mRNA is found in high concentrations throughout the epididymis, with a somewhat elevated concentration in the caput epididymal segment (523). Although 5α-reductase type 2 mRNA is present in abundance in the epididymis relative to the type 1 mRNA, its activity as a functional enzyme appears to be relatively poor. Unlike the developmental pattern of 5α-reductase type 1 mRNA expression, characterized by dramatic increases occurring just before the first appearance of spermatozoa in the epididymis, 5α-reductase type 2 mRNA expression does not show any significant developmental changes in any epididymal segment (523). Efferent duct ligation results in a dramatic decrease in 5α-reductase type 1 mRNA levels in the initial segment, but causes a near doubling in type 2 mRNA levels. This differential regulation of 5α-reductase type 1 and type 2 transcripts in one tissue has not been observed in other rat tissues such as the prostate (523).

In comparing the regulation of epididymal 5α-reductase enzyme activity and the expression of its mRNAs, it is apparent that a potential regulatory step is at the level of the protein itself. Post-transcriptional regulation has been proposed for several other epididymal proteins (79,135,345,466,529,530). In the adult rat, 5α-reductase type 1 protein expression is intensely immunolocalized in discrete lobules of the proximal initial segment of the epididymis; a sharp decline in staining intensity occurs between the proximal initial segment and its adjacent region, followed by a progressive decrease in intensity beyond that point [reviewed in Robaire and Viger (517)]. In all epididymal regions, 5α-reductase immunoreactive protein is localized specifically in the epithelial principal cells and is uniquely associated with membranous cytoplasmic elements; the intracellular localization of the 5α-reductase protein changes with movement down the epididymis. Savory et al. (1995) (531) have localized type 1 5α-reductase to the nuclear envelope of both transfected cells and rat tissues. The infranuclear localized form of the 5α-reductase type 1 protein is regulated in a similar fashion to the type 1 mRNA and enzyme activity, that is, by a paracrine factor of Sertoli cell origin (517,532).

The genomic regulation of the expression of both 5α-reductase isozyme genes is still poorly understood. Cloning and characterization of the proximal 2.2 kilobases of the 5′ upstream region of rat 5α-reductase type 2 have revealed that there are regions with strong repressor and enhancer activity; potential transcription factors (Sp1 and Sp3) regulating gene expression have been identified and immunolocalized to principal cells of the epididymis. The promoter region of rat 5α-reductase type 2 is GC-rich with a noncanonical TATA box located upstream of the

transcriptional start site (461). The potential role of this GC-rich region is intriguing in light of the observation that the methylation pattern of the two genes appears to differ not only between each other but between reproductive and nonreproductive tissues (533). The genes for 5α-reductase types 1 and 2 are also differentially methylated in lymphocytes from normal and 5α-reductase–deficient patients (534).

Estrogen Receptors and Cytochrome P450 Aromatase

Estrogen Receptors. Starting in the 1970s, substantial evidence accumulated for the direct action of estrogens on androgen target tissues. Using binding studies, specific, high-affinity cytosolic and nuclear estrogen-binding proteins, presumably receptors, were identified by a number of investigators in mice (535), rats (536–538), guinea pigs (539), rabbits (540–543), dogs (472,544), rams (545), monkeys (546,547), and men (548). Through a comprehensive series of studies, Danzo's group established the presence of estrogen receptors in the rabbit epididymis and demonstrated both its developmental (549) and regional distribution (539); however, enthusiasm for further studies on the estrogen receptor in the epididymis diminished when this same group demonstrated the presence of a protease, in the adult, that cleaves the DNA binding region of the estrogen receptor (550).

The histolocalization of tritiated DHT and estradiol in the adult mouse epididymis revealed differential distribution of grains over nuclei in different segments of the excurrent duct system, as well as over different cell types (535,551). These studies confirmed the earlier binding experiments and provided a more detailed localization of the putative receptors. Very strong labeling of the efferent ducts and initial segment of the epididymis was noted, with lesser binding in the more distal segments of the epididymis; nuclei of clear cells were more densely labeled with estradiol than with DHT, in sharp contrast to what was seen over nuclei of principal cells. Based on such data, and before the studies with null mutant mice, specific functions for estradiol in the epididymis were proposed (1,552).

With the discovery of the existence of two estrogen receptors (ERα and ERβ), and the availability of specific antibodies for these receptors as well as mice with null mutations for either or both receptors, our understanding of the localization of estrogen receptors and their potential role(s) has grown rapidly [reviewed in Hess (553)]. ERα and ERβ are remarkably similar to one another in some respects (e.g., 95% identity in the DNA binding domain), but they have individual characteristics (e.g., <55% homology in the ligand binding domain) that clearly indicate functional differences (554,555). The two isoreceptors have different binding affinity for estrogenic and antiestrogenic compounds (537,556), different tissue distributions (557), and different response elements (558,559).

Although there are some conflicting data on the immunolocalization of ERα and ERβ along the efferent ducts and the epididymis, a general pattern is emerging. In the mouse, the epithelium of the efferent ducts show strong ERα and weaker ERβ immunoreaction (553,560,561). The initial segment of the epididymis shows some reactivity for ERα, primarily in narrow, apical, and some basal cells (485); in the caput epididymidis, principal cells and clear cells stain positively, whereas in the distal regions only clear cells are immunoreactive. In contrast, ERβ immunoreactivity is clearly observed along the entire epididymis, with more intense staining in the more distal regions (corpus and cauda) (553,562). In the rat, in spite of some earlier controversies relating to the method of section preparation and antibody used for immunolocalization studies (551,563), it appears that ERα and ERβ immunolocalize in patterns similar to those of the mouse. The presence of ERα and the strong immunoreactivity of ERβ all along the epididymis have been confirmed using RT-PCR data (551,564) [reviewed in Hess (553)]. In monkeys, baboons, and humans, both forms of the estrogen receptor are present along the epididymis (565–568).

The first clear insight into the functional role of ERα in the efferent ducts and epididymis came through null mutation studies. The ERα knockout mouse was developed by Lubahn et al. (1993) (569) and is infertile in spite of the presence until puberty of apparently normal seminiferous tubules; in the adult, testicular atrophy becomes evident (570). Through an elegant series of studies, Hess's group (553,571,572) has demonstrated that the cause of infertility is back-pressure atrophy of the seminiferous tubules owing to the inability of the efferent ducts and initial segment of the epididymis to reabsorb the large volume of fluid secreted by the testis. This reabsorption process is under the control of estradiol, acting as a paracrine regulator of the efferent ducts. Further, it has been proposed that the action of estrogen on fluid reabsorption is mediated by the cystic fibrosis transmembrane conductance regulator (CFTR) receptor (573). In contrast to the clear role of the ERα receptor, function of ERβ in the male reproductive tract awaits further investigation because the ERβ knockout mouse is fertile and appears to have a normal testis and epididymis (574).

Cytochrome P450 Aromatase. An ongoing source of ligand is necessary to activate estrogen receptors. Cytochrome P450 aromatase, localized to the smooth endoplasmic reticulum, is a terminal enzyme that irreversibly catalyzes the conversion of testosterone to estradiol. Both the absence of aromatase and an

excess of estrogen result in male infertility [reviewed in Carreau (575)]. There are several sources of estradiol in the adult male reproductive tract. In the testis of adults, both Leydig cells (576,577) and germ cells (578) have been reported actively to synthesize estradiol. In germ cells, the enzyme is localized in the Golgi of round spermatids and throughout the cytoplasm of elongating and late spermatids [reviewed in Hess (553), Carreau (575), and O'Donnell et al. (579)].

In the epididymis, aromatization of the very high concentrations of testosterone to produce estradiol, particularly in the proximal segments, could occur either in the luminal or the epithelial compartments. The presence of cytochrome P450 aromatase in epididymal spermatozoa has been demonstrated in several species, including mouse, rat, bear, and rooster (578,580–582). The enzyme is localized to cytoplasmic droplets, and the staining becomes less intense as spermatozoa traverse the epididymis (583).

Early investigations of the presence of cytochrome P450 aromatase in epididymal epithelial cells indicated that this activity was absent (584); however, no direct assessment of the enzyme was done in these studies. In several subsequent investigations, direct detection of the P450 aromatase mRNA, protein, and enzyme activity (formation of estradiol from testosterone), as well as the ability of specific aromatase inhibitors to inhibit estradiol formation, all led to the conclusion that epididymal principal cells, but not the smooth muscle cells surrounding the duct, have the ability to synthesize estradiol from testosterone in a number of species, including the rat (585), monkey (586), and human (587).

Retinoic Acid Receptors. Retinoids are highly potent molecules with pleiotropic action in a wide range of biological processes during development and in the adult (588). Vitamin A deficiency results in both keratinizing and nonkeratinizing squamous metaplasia of numerous epithelia, including the epididymal epithelium (589,590).

Given the importance of retinoids, it is not surprising that a complex system has evolved to control retinoic acid biosynthesis and metabolism (591–593). Because retinoids are hydrophobic molecules insoluble in water, they require chaperones to ensure proper storage, transport, and uptake in the tissues. These chaperones, the retinoid-binding proteins, are members of either a large superfamily of small intracellular proteins that includes numerous fatty acid–binding proteins or another superfamily of secreted proteins, the lipocalins, which bind hydrophobic molecules. There are four intracellular retinoid-binding proteins that are members of the fatty acid–binding protein family (cellular retinol-binding protein [CRBP] I and II, and cellular retinoic acid–binding protein [CRABP] I and II), and two extracellular retinoid-binding proteins that are members of the lipocalin family (retinol-binding proteins and epididymal retinoic acid–binding protein [E-RABP]).

Within the nucleus, two classes of nuclear retinoic acid receptors, RAR and RXR, have been identified and cloned. Each receptor class consists of three receptor subtypes, α, β, and γ, and their isoforms. RXRs form heterodimers with RARs that are thought to be the functional units transducing the retinoid signal. The binding of RARs/RXRs to retinoic acid response elements modulates gene expression and is likely to be responsible for the observed biological effects of retinoids (594).

Most of the components of the retinoid signaling pathway have been identified in the epididymis. Retinoids were quantified in the rat epididymis by high-performance liquid chromatography (HPLC) followed by derivation of the HPLC-purified retinoids to confirm their identities (595). In the epididymal tissue, the major retinoids identified included retinol, retinyl ester, all-*trans* retinoic acid, and 9-*cis*-retinoic acid (9-*cis*-RA). The concentration of retinol and retinyl esters decreased from the caput to the cauda epididymidis from 2.7 to 1.7 nmole/g of tissue and from 6.2 to 1.4 nmole/g tissue, respectively. The active retinoids, all-*trans* retinoic acid and 9-*cis*-RA, showed an inverse gradient, with lowest concentrations in the caput (all-*trans* retinoic acid: 13 pmole/g tissue; 9-*cis*-RA: 2.9 pmole/g tissue) and highest concentrations in the cauda (all-*trans* retinoic acid: 35 pmole/g tissue; 9-*cis*-RA: 6.8 pmole/g tissue). Using a bioassay, levels of 7 pmole/g of tissue have been reported in the murine epididymis (595a).

The CRBPs and CRABPs are also present in the epididymis, but their localization is regionalized. CRBP is most abundant in the columnar epithelial cells of the proximal caput epididymidis of the rat; this tissue has one of the highest CRBP expressions in the body (596–598). The mouse has the highest concentrations of CRBP in the proximal caput epithelium, but also has significant levels in the epithelium throughout the epididymis. CRABP II is found in the epithelium of the cauda epididymidis coexpressed with CRBP in both the mouse and the rat, whereas CRABP I is present in the smooth muscle of the distal cauda epididymidis (599).

The retinoid signaling pathway in the epididymis includes the retinoic acid receptors and the retinoic acid carrier protein E-RABP, synthesized and secreted into the lumen in the distal caput and found in the lumen from the caput to the cauda epididymidis. In the rat epididymis, RARα, β, and γ transcripts have been detected by RT-PCR (600). In situ hybridization and immunolocalization revealed region-specific

patterns of RARα mRNA and protein expression (601). The highest levels of RARα transcripts and RARα immunostaining were seen in the proximal caput epididymidis. The levels of RARα transcripts and RARα protein staining were higher in the initial segment, proximal caput, and distal cauda. The lowest levels were seen in distal caput, corpus, and proximal cauda epididymidis.

It is clear that a remarkable retinoid homeostasis system has evolved to permit the transport of these hydrophobic molecules and their metabolism, extracellular and intracellular sequestration, and delivery to the nuclear chromatin for optimum retinoid signaling in the epididymis. However, knowledge of the pattern of expression of the various components of the retinoid biosynthesis and metabolism pathway, although suggestive, is not sufficient to deduce the precise role of these regulatory factors or the site of action of retinoic acid in the epididymis. Vitamin A deficiency has pervasive effects, affecting many organs during prenatal and postnatal development and adult life. Reproduction is affected both at the level of the testis, where spermatogenesis is arrested, and at the level of the epididymis, where the epithelium develops squamous metaplasia. Because of this dual effect, it is difficult to ascertain from vitamin A deficiency studies the physiological role of retinoids in epididymal function.

Gene inactivation has produced surprising results. The transthyretin null mutant has a virtual loss of serum retinol-binding protein, yet the mice seem normal (602). CRABP I and CRABP II null mutant mice also appear normal (603,604). The double knockouts of CRABP I and CRABP II have a higher death rate at 6 weeks than the wild-type mice, but the survivors appear normal (604). Although each retinoid-binding protein separately appears to be dispensable, the lack of phenotype may simply reflect that redundant mechanisms are built into the organism.

The functional redundancy is well illustrated in the case of the retinoic acid nuclear receptors, of which six RXR isoforms and six RAR isoforms have been identified (605). Null mutations of RARβ, $RAR\alpha_1$, $RAR\beta_2$, or $RAR\gamma_2$ appear normal. Null mutations of all RARα isoforms or all RARγ isoforms display some of the defects of the postnatal vitamin A deficiency syndrome. Only double-null mutants of the RAR subtypes (RARα/γ, $RAR\alpha/\beta_2$, $RAR\alpha_1/\beta_2$, $RAR\beta_2/\gamma$) reproduce most of the features of vitamin A deficiency syndrome. Null mutation of RXRα induces some abnormalities, but double inactivation of RXRα and RARβ or RARγ increases the severity of the RXR phenotype. It appears that even though the different receptor isoforms are not truly functionally equivalent, they can substitute for one another under certain conditions. This functional redundancy may not be surprising in any gene family that has been conserved throughout vertebrate evolution and has evolved by duplications from an ancestral gene.

Even with these caveats, an epididymal phenotype has been observed in transgenic mice after disruption of the RAR gene. Expression of a dominant negative mutant of RARα induced loss of organization of the columnar epithelium lining the cauda and its transformation by squamous metaplasia. The aberrant transformation resulted in blockage or rupture of the duct with concomitant inflammatory response and, ultimately, infertility (606). The RARα null mutant mice display not only aspermatogenesis, but vacuolization of the epididymal epithelium (607), and the RARα/γ double-null mutants display either severe dysplasia or complete agenesis of the epididymis and the vas deferens (608).

In summary, given the pathological changes seen in the epididymal epithelium in vitamin A deficiency, the epididymal phenotypes seen in RAR null mutant mice, and the presence of most of the components of the retinoid signaling pathway, it is highly likely that retinoids play a regulatory role in epididymal function.

Oxytocin Receptors. The first demonstration of the presence of oxytocin receptors in the epididymis was by Maggi et al. (1987) (170). Since then, several reports on the localization, distribution, regulation, and potential functions of these receptors in a variety of species have appeared (169,171–173).

Although in the marmoset monkey, the oxytocin receptor seems to be primarily expressed in peritubular cells of the epididymis, in the human, macaque monkey, and ram, its localization is in the epididymal epithelium and varies along the duct (169,172). The expression in principal cells is seen only in the initial segment of the epididymis of primates and has a checkerboard appearance; throughout the rest of the duct, it is confined to peritubular cells of the epididymis (172). In the ram, however, oxytocin receptors are found in both principal and basal cells throughout the epididymal duct, whereas peritubular staining is seen only in the more distal segments (173).

Two functions have been ascribed to the oxytocin/-oxytocin receptor system in the epididymis. As discussed previously, the first is that of promoting contractility of the duct, as demonstrated by both in vitro (174) and in vivo (179) studies, thus promoting sperm transport through the epididymis (187). The second function of this system involves the promotion of the formation of DHT by stimulation of 5α-reductase activity in the initial segment of the epididymis (609). This function is of particular interest, given the very high 5α-reductase enzymatic activity in this segment of the epididymis and the presence of oxytocin in the luminal fluid of the ram and the rat (610,611). The mechanism for this type of stimulation

is not clear but has been postulated to be mediated by phosphorylation and activation of existing 5α-reductase due to tyrosine kinase activation through a coupling to the oxytocin receptor by G_i signal transduction (612).

Growth Factor Receptors. FGF receptors (FGFR), platelet-derived growth factor (PDGF) receptors, and vascular endothelial growth factor (VEGF) receptors have all been identified in the epididymis. For FGFR, two splice variants, FGFR-1 IIIc (long and short forms), have been identified in the principal cells of the initial segment (459). Although all four major splice variants of FGFR (1 through 4) are present in initial segment tissue, only the FGFR-I IIIc variants are present in principal cells. The remaining splice variants are presumably localized to the endothelial and interstitial cells. FGFs 2, 4 and 8, present in rete testis and epididymal luminal fluid (458,459), are potential ligands for the apically placed receptor (S. Crenshaw and B. T. Hinton, unpublished observations).

PDGF α and β receptors and their ligands (PDGF-A through D) have been localized in the rat and mouse epididymis (613). In view of the specific localization of PDGF-A in the epithelium and the PDGF α receptor in the mesenchyme, the authors suggested that the PDGF-A/α receptor system may be involved in epithelial–mesenchymal interactions during epididymal development. In the adult epididymis, PDGF-A and B and α and β receptors were identified in the epithelium only. Interestingly, in PDGF-A null mutants, an epididymal phenotype was not observed during development, but epididymal epithelial disruption was observed at postnatal day 25 (613).

VEGF and its receptors are highly localized in both the testis and epididymis, with fms-like tyrosine kinase (flt-1) and fetal liver kinase (designated as kinase insert domain–containing receptor [KDR]) VEGF receptors being identified by immunohistochemistry in the ciliated cells of the efferent ducts and vascular endothelial cells, respectively, of the human epididymis (614). Flt-1 was also observed in the lymphatics. Furthermore, addition of VEGF28 to epididymal tissue in culture resulted in endothelial fenestrations and opening of interendothelial junctions. Because the ligand, VEGF, was localized to the peritubular and ciliated cells of the efferent ducts and to the peritubular and basal cells of the epididymis, it was suggested that it may play a paracrine role in the permeability of blood vessels through KDR and lymphatics through flt-1 (614). Overexpression of VEGF in the testis and epididymis results in male infertility due to spermatogenic arrest and increased capillary density in the testis and epididymis (615).

Although not specifically expressed in the epididymal epithelium, the hepatocyte growth factor receptor, c-met, is localized to epididymal spermatozoa, with the ligand, hepatocyte growth factor, being secreted into the lumen by the epididymal epithelium. The putative action of hepatocyte growth factor is maintenance of sperm motility (616). Interestingly, the hepatocyte growth factor–like protein receptor, Ron mRNA, which is a receptor tyrosine kinase, is expressed in the epididymal epithelium (617). Additional growth factor–binding proteins and growth factor receptors that are expressed in epididymal epithelial cells include insulin-like growth factor–binding protein-rP1/mac25 (618), latent transforming growth factor-β–binding protein 2 (619), epidermal growth factor receptor (620), and growth hormone receptor–binding protein (621).

c-Ros. c-Ros is an orphan tyrosine kinase receptor that has received considerable attention since it was shown that c-Ros null male mice were infertile (40). The kinase and extracellular domains of the *c-Ros* gene show similarities to the *Drosophila sevenless* (*sev*) tyrosine kinase receptor (622). The *sev* gene was originally discovered in a *Drosophila* mutant that failed to express the R7 photoreceptor cell in the ommatidium (623,624). Further studies demonstrated that the adjacent R8 photoreceptor cell expressed *boss*, the ligand for sev. Interaction between these two cell types was crucial for the development of these photoreceptor cells. After this cell interaction, a signal transduction cascade involving Ras and activation of downstream target genes was initiated in the R7 photoreceptor cell. Interestingly, ectopic expression of *boss* or of constitutively active forms of *sev* causes the formation of ectopic R7 cells (625,626). The overall sequence homology between *c-Ros* and *sev* is quite low, but there are some very interesting structural similarities between the two. For example, (a) both genes encode a large extracellular domain and a kinase domain; (b) sequence homology extends into the putative extracellular ligand-binding domain; (c) there is a short insert of five to seven amino acids in the same position in the kinase domain of both genes that is not found in other receptor tyrosine kinases; (d) there are several common exon–intron junction points between the two genes; (e) the kinase domains of both genes are 70% homologous (622,627–630); and (f) particularly striking is the analysis of Springer (1998) (631), who showed a colinearity and spacing of β-propeller and epidermal growth factor-repeat structures in c-Ros and *sev*. Interaction of ligand with β-propellers has been shown for other proteins (632). The ligand of sev is bride of sevenless (boss) (626,633), a seven-transmembrane G-coupled protein kinase, which has

signal transduction properties of its own. Presumably, a *boss* homolog exists in mammals if *c-Ros* has a similar function to *sev*.

Although c-Ros is expressed in several tissues throughout development, including the lung, kidney, and epididymis, it remains highly expressed in the initial segment of the adult mouse epididymis (40,634). A clue to the function of c-Ros came from c-Ros knockout mice: the animals displayed an underdeveloped initial segment and male infertility. The infertility phenotype was due to spermatozoa having an angulated flagellum at the end of the midpiece (635); it was suggested that this defect was caused by the failure of spermatozoa to regulate their cell volume (635). This infertility phenotype was also shown to be cell autonomous; the defect in the sperm phenotype was due to the passage of normal spermatozoa through the underdeveloped initial segment, and not to some inherent defect in the spermatozoa themselves. Further, the infertility phenotype was observed if either the kinase domain or the extracellular domain of c-Ros was lacking (40).

Although the functional role of c-Ros is not known, it has been suggested that it plays a role in the regulation of cell volume (636,637), and although c-Ros itself may not have a direct effect on cell volume regulation, gene expression profiling suggests that several osmolyte transporters are compromised in the initial segment of the c-Ros null animals (467). It also appears that at least inositol and glutamate sperm concentrations are lower in the c-Ros knockout mice compared with controls, even though there are no differences in luminal fluid concentrations of the two osmolytes between the two groups (377). Whether the lower intracellular levels of these osmolytes in spermatozoa is the cause of the infertility phenotype remains to be determined. Adding to the complexity of the problem is that the infertility phenotype may be related to an underdeveloped initial segment rather than directly to c-Ros. Because the adult mouse initial segment still expresses c-Ros, the challenge will be to silence this gene in the adult and then examine the fertility phenotype. This experiment needs to be done, especially if c-Ros and its downstream signaling pathways are to be considered as potential targets for the development of a male contraceptive. An additional important finding is that the adult human epididymis also expresses c-Ros along its length (638).

In an attempt to identify downstream targets of c-Ros, Keilhack and colleagues (2001) (639) used a yeast two-hybrid assay and identified several proteins that associated with the kinase domain of c-Ros. These included the phosphatase SHP-1 (Src homology region 2–containing protein tyrosine phosphatase-1), phospholipase Cγ, the SH2 domain of c-Abl, Grb2, the phosphoinositide-3 kinase subunit p85α and the SH2 domains of SHP-2. To demonstrate the potential importance of the SHP-1 phosphatase in male fertility, *viable motheaten* (me^v) mice were tested for fertility because these mice contain a mutation in the SH2 domain of SHP-1. The male mice were infertile. Hence, downstream targets of c-Ros are clearly excellent candidates for male contraceptive development. Although Keilhack et al. (2001) (639) claim that the infertility phenotype is similar to that seen in the c-Ros knockout animals, the authors do not present convincing data that there is a reduction in epithelial cell height in the initial segment of the SHP-1 knockout animals, similar to that seen in the initial segment of c-Ros knockout mice. Their data suggest that the initial segment has developed normally in this knockout. Hence, although male infertility is observed in both the c-Ros and SHP-1 knockouts, the mechanisms by which infertility is achieved in each case are probably different.

HE6. The HE6 gene encodes a novel human epididymal protein that comprises a seven transmembrane component with a large extracellular domain and shows high homology to the secretin/pituitary adenylate cyclase–activating polypeptide (PACAP) superfamily of G-protein–coupled receptors (GPCRs) (640). In view of the large ectodomain, HE6 has also been considered to be a member of the LNB-TM7 proteins (family-B GPCR–related TM7 receptors with long N-terminal extensions) (641). This large domain also contains a GPCR proteolysis site, and therefore HE6 may exist as a two-subunit receptor. HE6 is located on the human X chromosome, and both rat and mouse homologs of HE6 have been cloned (640,641). Immunolocalization studies show human and mouse HE6 localized to the apical surface of human epididymal cells, with staining more pronounced in the efferent ducts–caput region compared with the distal epididymal regions. A mosaic pattern of staining was also observed, as is often seen with localization of other epididymal proteins. Mouse HE6 was localized to the apical cell surface but highly restricted to the initial segment region (641). The function of HE6 is yet to be determined, although a knockout study suggests that the protein may be involved in fluid reabsorption in the efferent ducts (642).

Other Receptors. In addition to those receptors discussed in the preceding sections, the epididymis also expresses orexin receptors (643–645), the urokinase-type plasminogen activator receptor (646), apolipoprotein E receptor-2 (647), adrenomedullin receptors (648), purinergic receptors (649), and guanylyl cyclase C receptor (650).

Transcription Factors

An understanding of transcriptional regulation of epididymal genes has been limited to a few genes [reviewed in Robaire et al. (90), Cornwall et al. (91), Kirchhoff (92), Rodriguez et al. (651), Rao and Wilkinson (652), and Suzuki et al. (653)]. However, in these studies, several key transcription factors have been identified that may play an important role in the tissue-specific, region-specific, or cell-specific expression of epididymal genes. For the sake of simplicity, steroid hormone receptor coregulators are not discussed here, but are discussed in the section on Receptors and Their Ligands.

PEA3 Family. One of the first major epididymal transcription factors to be examined was polyomavirus enhancer activator 3 (PEA3), which is a member of the Ets transcription factor family. This family is characterized by an 85–amino acid Ets domain (654) that encodes for a helix-turn-helix DNA binding motif. It is also part of a subfamily, referred to as the PEA3 family, that comprises PEA3, ERM, and ER81 (655–660). The consensus site for PEA3 is 5′-AGGAAG-3′, with GGAA being the core binding motif for the Ets family. Xin et al. (1992) (655) were the first to show expression of PEA3 in the epididymis, and later studies by Lan et al. (1997) (460) and Drevet et al. (1998) (661) clearly showed a role for members of the PEA3 family in regulating GGTIV and GPX5 gene expression in the epididymis. Standard in vitro promoter analyses of both GGTIV and GPX5 revealed that PEA3 acts as both a repressor and an activator of transcription. This was confirmed more recently by Kirby et al. (2004) (662) using a novel in vivo electroporation technique. There were differences in promoter activity between the in vivo and in vitro studies when the same constructs were used, highlighting the importance of performing such an analysis under as near-physiological conditions as possible. The arrangement of PEA3 binding sites along the GGTIV promoter may be unique to this gene because of the absence of binding motifs (e.g., AP-1) that are known to regulate the activity of PEA3 in many other gene promoters (e.g., urokinase plasminogen activator and matrix metalloproteinase) (663,664).

There appears to be redundancy among the PEA3 family members because SouthWestern analysis (460) showed that multiple proteins identified as PEA3, and possibly ERM and ER81, bound to GGTIV mRNA PEA3 binding motifs. Further, PEA3 knockouts had a normal epididymal phenotype, supporting the idea that ERM and ER81 may take over the functions of PEA3. PEA3 knockout male mice are infertile, but their infertility is due to impotence or failure to ejaculate spermatozoa (665). Interestingly, the promoters of several genes that are highly expressed in the initial segment, including 5α-reductase type 2 (461), have PEA3 family binding motifs, and each gene, including PEA3 family members, is regulated specifically by testicular factors. It will be important to generate initial segment-specific PEA3 family member knockouts to determine whether PEA3 family members play an important role in the function of the initial segment and hence male fertility.

CCAAT/Enhancer-binding Protein β. CCAAT/enhancer-binding protein β (C/EBPβ) is a member of the CCAAT/enhancer-binding proteins that regulate genes that have various functions, including cell cycle control, fatty acid metabolism, cellular differentiation, growth, tumorigenesis, and apoptosis [reviewed in Ranji and Foka (666)]. This six-member transcription factor family is characterized by a highly conserved basic-leucine zipper domain at the carboxyl terminus that is involved in dimerization and DNA binding. Interestingly, although heterodimerization can occur, the heterodimer C/EBPβ-C/EBPζ does not bind to the standard C/EBP consensus sequence, but binds to a subset of promoters in genes involved in cellular stress. Hence, this family of transcription factors can function as either repressors or enhancers depending on the physiological state of the cell (667).

C/EBPβ is expressed in the proximal regions of the mouse epididymis and plays a role in the regulation of expression of the CRES (cystatin-related epididymal spermatogenic) gene (668). Two putative C/EBP DNA binding motifs were identified in the first 135 base pairs of the CRES promoter. Data from gel shift assays, in vitro and in vivo promoter analyses, and analysis of C/EBPβ knockout mice suggest that expression and activation of C/EBP is necessary for complete activation of CRES in the proximal caput region (662,668). Further analysis showed that although C/EBPβ was the predominant form expressed in the epididymis, C/EBPα and δ were also expressed, but to a lesser degree. C/EBPε was not identified (668). Because the expression of members of the CRES gene family depends on the presence of testicular luminal fluid factors (669), it is assumed that C/EBP family members are also regulated in a similar manner. Because members of C/EBP family play a role during cellular stress, it would be interesting to examine their potential role during apoptosis of the initial segment after loss of testicular factors.

Homeodomain-containing Transcription Factors. Several classes of homeodomain-containing proteins are expressed in the mammalian epididymis; these include hox (homeobox), PEPP-family (PEM), Prd-class, and Pou-class genes [reviewed in Rao and Wilkinson (652) and Bomgardner et al. (670)]. Homeodomain-containing proteins are transcription factors that contain a highly conserved 60–amino acid

DNA binding domain composed of three α-helices, with the third helix interacting closely with DNA. The major role of hox genes is in organ development and the establishment of segment identity along the anteroposterior body axis (671,672). The epididymis is a highly segmented organ, and therefore it is not surprising that many hox genes are expressed in the developing epididymis (670,673–675). Because the epididymis forms later in development, it is the 5′ hox genes that appear to play a major role in epididymal development compared to the earlier expressed 3′ hox genes. Interestingly, hox genes are also expressed in the adult epididymis (675); their precise function in the adult warrants further study.

A first indication that hox genes were expressed in the epididymis and played a role in epididymal development came from an analysis of reproductive tissues from hoxa10 and hoxa11 knockout animals (673,676). One of the most intriguing phenotypes to be produced from these knockouts was the homeotic transformation of the distal epididymal regions. Hoxa11 was expressed in the initial segment and in the more distal epididymal regions (675), so the observation of a distal epididymal phenotype in the hoxa11 knockout was not surprising. However, it is not clear if the epididymis developed appropriately in this mutant because the initial segments of these animals were not analyzed. The regulation of hox genes in the epididymis is poorly understood; however, it is known that in many tissues cofactors must be involved in the transcriptional activation of genes by hox transcription factors. Two examples include Meis family members and PBX (677), both of which contain a homeodomain; Meis 1a and 1b are expressed in the mammalian epididymis (675).

More recently, a new homeobox cluster termed *Rhox* has been identified on the X chromosome. Although most of the members of this family are expressed in the testis, several are also expressed in the epididymis (678). A putative homeodomain transcription factor called Phtf1 has been identified in the principal cells of the initial segment and caput region (679). Although this protein contains domains that suggest it is a transcription factor, immunolocalization studies show it to be localized to the endoplasmic reticulum saccules applied to the *trans* face of the Golgi system.

One the best-studied homeodomain-containing transcription factors is PEM, a member of the PEPP homeobox subfamily. Rao and Wilkinson (2002) (652) suggested that the PEPP genes were derived from the Drosophila *aristaless* gene. Of the PEPP gene family members, only PEM was expressed in the epididymis as well as in the testis (652). PEM, like hoxa11, was expressed throughout development and into adulthood (680,681) from a "male-specific" proximal promoter (Pp). The expression of PEM in the epididymis was species dependent. In the mouse, PEM was found mainly in the caput, whereas in the rat it is expressed throughout the duct, increasing from caput to cauda. However, PEM was not expressed in the initial segment of either the rat or mouse (652). Further, expression of PEM was androgen dependent (680,682). The function of PEM is not known because PEM knockout animals did not display an epididymal phenotype (683), although Rao and Wilkinson (2002) (652) suggested that the function of PEM may be context dependent (e.g., under different environmental conditions).

Other transcription factors that have been identified in the epididymis include sp1 and sp3 (461,684), c-Fos (91), Tst-1 (685), Emx-2 (686), Lbx-2 (687), pax-2 (688), zfy (689), and nor-1 (690).

Transporters and Channels

Water and Ion Transport. Although the efferent ducts serve as a conduit for spermatozoa from the testis to the epididymis, a major function of the efferent ducts is the reabsorption of water. The major driving force for water movement across the efferent ducts and the cauda epididymidis is the Na^+/K^+-ATPase located on the basolateral membrane (691,692). However, because the activity of the Na^+/K^+-ATPase in the efferent ducts is less than that in the cauda of the epididymis, the paracellular pathway also plays a critical role in sodium and water movement (356) across the epithelium of the efferent ducts.

The involvement of an apically placed Na^+-H^+ exchanger that drives sodium out of the lumen with water following, was proposed by Wong and colleagues (2002) (358) because fluid absorption is amiloride inhibitable, isosmotic, and flow dependent. So far, four Na^+-H^+ exchangers (NHE1 through 4) have been identified in several tissues, and three of them have been found in the epididymis (693–696); such transporters appear to play a role in sodium and bicarbonate reabsorption and proton secretion. NHE1 and NHE2 are expressed on the basolateral surface and apical surface, respectively, throughout the epididymal duct, except that NHE2 is not expressed in the initial segment. Cheng-Chew et al. (2000) (694) suggested that NHE1 is involved in bicarbonate secretion, whereas NHE2 may be involved in sodium reabsorption. Studies by Breton et al. (695) and others (693,696) have shown that the expression of NHE3 varies throughout the length of the epididymis and is localized to the apical surface of principal cells only. Important to bicarbonate reabsorption is the presence of apical and cytoplasmic carbonic anhydrases. NHE3 appears to participate in bicarbonate

reabsorption, similar to its role in the renal proximal tubule. Through the activity of apically placed NHE3, protons are secreted and sodium is reabsorbed. Carbonic anhydrase IV, which is also apically positioned, then converts the luminal protons into water and carbon dioxide, allowing the carbon dioxide to enter the cells. Cytosolic carbonic anhydrase II catalyzes the production of new intracellular bicarbonate and generates a proton. The proton is recycled back into the lumen by NHE3. Intracellular bicarbonate is then transported out of the cell across the basolateral membrane by the bicarbonate exchanger (AE2) (473), or the electrogenic sodium-bicarbonate cotransporter, NBC1 (473). The driving force for this whole process is provided by the low intracellular sodium concentration achieved by the basolateral Na^+/K^+-ATPase. In addition, maintenance of a high potassium concentration by the Na^+/K^+-ATPase induces an intracellular negative potential of approximately −70 mV. This drives bicarbonate across the basolateral membrane through the NBC1 transporter, which transports three molecules of bicarbonate for every sodium molecule, resulting in the net transfer of two negative charges.

A major player in hydrogen ion secretion is vacuolar-type H^+-ATPase, which is highly expressed in apical/-narrow cells in the proximal regions of the epididymis and in the clear cells throughout the epididymis (97,697–700). Using a proton-selective electrode, Breton and colleagues (1996) (699) showed that the epithelium of the vas deferens secreted protons and that this was inhibited by bafilomycin, an inhibitor of H^+-ATPase. This study clearly demonstrated that acidification of the luminal fluid in the vas deferens was due to this transporter. To maintain an acidic luminal fluid microenvironment, clear cells probably respond to a luminal increase in bicarbonate by increasing their rate of proton secretion (106). Further, the rapid recycling of the H^+-ATPase in clear cells suggests that the regulation of proton secretion may involve unique clathrin- and caveolin-independent mechanisms, as well as members of the SNARE protein family; using colchicine, a microtubule network has been found to be important [reviewed in Breton (360)].

The aquaporins also play a role in water reabsorption and, of the 10 isoforms, AQP1, 2, 3, 8, and 9 have been identified throughout the epididymis, including the efferent ducts (701–708), but not necessarily in every region and epithelial cells. Although very few functional studies have been done on the aquaporins, studies by Wong and colleagues (707) have shown that the cystic fibrosis transmembrane conductance regulator (CFTR) potentiates water permeability of AQP9 in oocytes and in the rat epididymis. CFTR is expressed in the rat and human epididymis. Besides CFTR's known role as a cAMP-activated chloride channel, its role as a regulator of AQP9 may have ramifications in men with cystic fibrosis (707). Other chloride channels have received little attention. However, a study by Isnard-Bagnis et al. (2003) (107) showed that the epididymis expresses members of the CIC gene family of chloride channels. Studies from this group suggest that CIC-3 may be involved in chloride transport by principal cells, and CIC-5 might play a role in the acidification of H^+-ATPase–containing vesicles in narrow and clear cells.

Although net water movement is from lumen to cell, water is still secreted and plays an important role in ion and water homeostasis. Chloride and bicarbonate are again responsible for driving water from the cell into the lumen, and Wong and colleagues (2002) (358) have undertaken extensive investigations recognizing the importance of peptide hormones in this process. These investigators have presented a novel model to explain anion secretion by multiple peptide hormones. For example, angiotensin II, bradykinin, endothelin, and arginine vasopressin interact with their cognate receptors on the basolateral membrane, which in turn activates phospholipase A2 on membrane phospholipids, resulting in the release of arachidonic acid. PGs are subsequently produced through the actions of COX-1 and isomerases. This process occurs in the basal cells. PGs then diffuse out of the basal cells and interact with their receptors on the basolateral membranes of principal cells, which in turn causes an increase in cAMP resulting in activation of the apically placed CFTR channel and chloride secretion (Wong et al., 2002) (358). With the identification of the family of Trp channels in the epididymis, Leung et al. (2004) (116) suggest that Trp3 provides the increase in intracellular calcium levels, which translocates phospholipase A2 from the cytosol to membrane phospholipids, where COX-1 has been identified (Fig. 15).

In summary, water secretion is driven by chloride transport, whereas fluid reabsorption is principally driven by sodium transport. As suggested by Wong and colleagues (2002) (358), secretion of water may play a role in fine-tuning water movement across the epididymal epithelium. This may be particularly important because for now very few hormones appear to play a role in regulating water reabsorption. However, estrogen has been implicated as a major regulator of water reabsorption in the efferent ducts (551,571,709). As discussed previously, the efferent ducts in an ERα knockout mouse failed to reabsorb water sufficiently, resulting in the accumulation of fluid and spermatozoa, testicular blockage, and atrophy. Later studies by Hess and others suggest that androgens may also play a role in the regulation of water reabsorption by the efferent ducts (553). If hormones such as estrogen and androgen play a

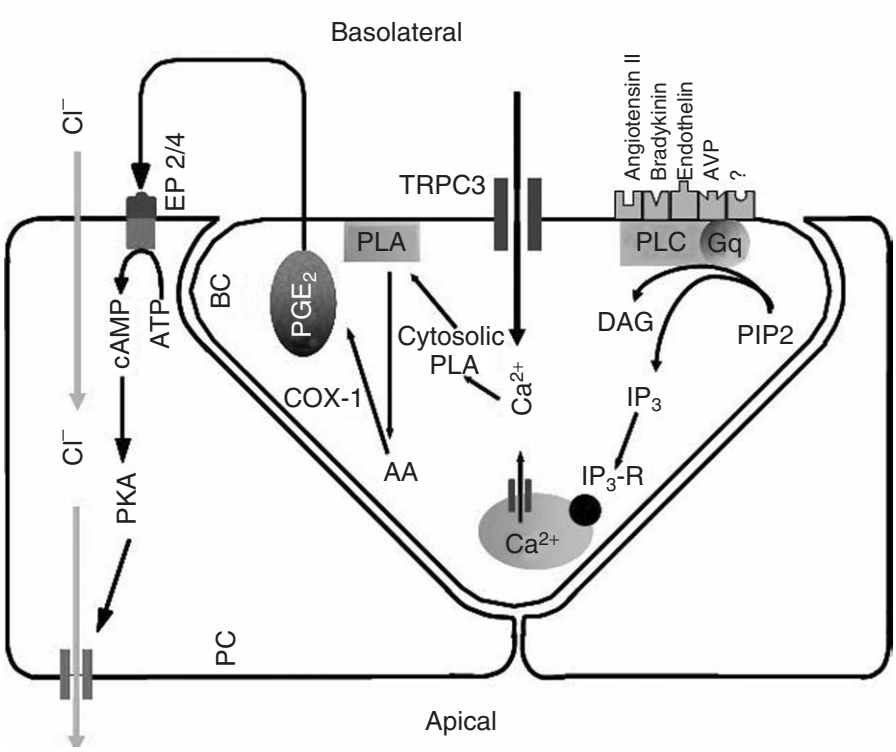

FIG. 15. Schematic diagram illustrating the roles of cyclooxygenase (COX)-1 and transient receptor potential 3 (Trp3) in the regulation of principal cell functions by basal cells in the rat epididymis. (Modified from Leung, G. P. H., Cheung, K. H., Leung, T., Tsabg, M. W., and Wong, P. Y. D. [2004]. Regulation of epididymal principal cell functions by basal cells: role of transient receptor potential [Trp] proteins and cyclooxygenase-1 [COX-1]. *Mol. Cell. Endocrinol*. 216, 5–13; kindly supplied by Dr. Patrick Wong.)

role in water reabsorption in the efferent ducts, it will be of interest to identify their downstream targets to uncover the mechanisms by which these hormones regulate water movement.

Organic Solute Transporters. Few studies have focused on the physiology of transport, although some organic transporters, such as ion transporters, have been identified in gene arrays (353,467–468a). This aspect of the review focuses only on those transporters that have been identified as functioning in the epididymis.

L-Carnitine Transporters. In 1998, two independent laboratories reported the cloning of a high-affinity L-carnitine transporter, OCTN2, isolated from human and rat small intestine complementary DNA (cDNA) libraries (710,711). OCTN2 is a member of the organic cation transporter family. Thus far, only three members of this family, OCTN1, OCTN2, and OCTN3, have been shown to transport L-carnitine. OCTN2 transports L-carnitine against a concentration gradient in a sodium-dependent manner. In contrast, OCTN1 is a low-affinity, sodium-independent carnitine transporter (712,713), and OCTN3 is a high-affinity, sodium-independent carnitine transporter (714,715).

The OCTN2 cDNA encodes a 557–amino acid protein with a predicted molecular mass of 63 kDa. Mutations in OCTN2 are responsible for primary carnitine deficiency, an autosomal recessive disorder characterized by cardiomyopathy and muscle weakness (716–718). Hence, OCTN2 is a key protein involved in L-carnitine transport. OCTN2 is localized to the basolateral membranes of the rat epididymis in a region-specific manner, with highest mRNA and protein expression observed in epididymal regions involved in L-carnitine transport (392). Therefore, OCTN2 is the prime candidate for transporting L-carnitine into epididymal cells.

The function of L-carnitine in the epididymis is unknown, but the hypothesis that OCTN2 plays a role in protection against osmotic stress in the epididymis is supported by an intriguing observation. Toshimori et al. (1999) (719) described an animal model for primary carnitine deficiency, the juvenile visceral steatosis (*jvs*) mouse. These mice exhibit symptoms similar to those of patients with primary carnitine deficiency. Studies by Lu et al. (1998) (720) demonstrated that mutations in OCTN2 were responsible for the *jvs* phenotype, and complementation with human OCTN2,

but not OCTN1, rescues the phenotype in the *jvs* mice (721). Loss of OCTN2 results in the loss of integrity of the epididymal epithelium in *jvs* mice, especially in the corpus region, a site known to express high levels of OCTN2 and that readily transports L-carnitine. Therefore, it would seem plausible that loss of epididymal epithelial integrity in *jvs* mice may be the result of osmotic stress.

CT2, the putative protein that transports L-carnitine from cells into the lumen, has been identified (393). Although initially thought to be a testis-specific transporter, immunohistochemical studies clearly demonstrate its presence on the apical surface of human epididymal cells (393). The transporting characteristics and kinetics of CT2 are similar to those previously reported for an apical L-carnitine transporter in the rat (722); we propose that they are, in fact, the same transporter. CT2 is a somewhat unusual in that it can transport L-carnitine in either direction; transport partially depends on the presence of sodium (in both directions), and the transporter has limited substrate specificity (393). CT2 may have multiple functions despite its narrow substrate specificity. Certainly, from the distal caput to proximal cauda epididymidis, it functions to transport L-carnitine from cells into the lumen. However, CT2 can also transport solutes from the lumen into cells (722), suggesting that it ensures that any L-carnitine that does enter the lumen proximal to the distal caput is rapidly removed or that it can transport other solutes yet to be identified. Studies have revealed that flipt2 is the mouse homolog of human CT2, and that the mRNA and protein are expressed throughout the length of the mouse epididymis (B. T. Hinton, unpublished observations).

Taurine Transporters. Although not shown specifically to transport taurine into the epididymis, the taurine transporter TauT and the putative taurine channel phospholemman are expressed in the epididymis, with the more distal epididymal regions showing higher expression (723). Although these studies were performed using the mouse epididymis, these data coincide well with the measurement of intraluminal concentrations of taurine in the rat epididymis; the values measured were corpus (~6 mM) > cauda (~3mM) > caput (~2 mM) (396).

Glutamate Transporters. Expression of glutamate transporter EAAC1 and the glutamate transporter–associated protein (GTRAP) has been identified in the epididymis (724). Although the expression of both of these genes is high in the caput region, where the highest concentration of glutamate is observed [~50 mM (396)], expression is also high in the distal epididymal regions. This suggests that the protein is transporting glutamate into the lumen in the proximal epididymal regions, that the protein is transporting glutamate out of the lumen in the distal epididymal regions because the intraluminal concentration of glutamate rapidly declines from the caput to cauda epididymidis (396), or that the protein may be transporting other amino acids. Because many amino acid transporters are not specific for individual amino acids and some are bidirectional, all three possibilities are plausible. Definitive functional studies are needed to confirm that either EAAC1 or GTRAP (or both) is responsible for the high intraluminal concentrations of glutamate. An alternative possibility for the high concentrations of glutamate is the metabolism of intraluminal glutathione by γ-glutamyl transpeptidase (335).

CE11. More recently, a transporter called CE11 has been identified in the canine epididymis. This 12 transmembrane cotransporter has homology to the thymic stromal cotransporter (TSCOT), with some similarity to a sugar transporter (725). The transporter has been localized to the apical membranes of the canine epididymis and its function is not known.

Antimicrobial Peptides

Groups of epididymal proteins belonging to the defensin and defensin-like family and other proteins having antimicrobial properties have been identified in the epididymis. The presence of antimicrobial peptides in both reproductive and nonreproductive tissues has long been acknowledged, but more recently several antimicrobial peptides have been shown to be epididymis specific. Some of these are members of the β-defensin family, β-defensin–like peptides (e.g., bin1b, EP2, and HE2 variants), lactoferrin, cystatins, Eppin, and the cathelicidins (human cathelicidin antimicrobial protein-18 [hCAP18], cathelin-related antimicrobial peptide [CRAMP]) (726–745).

The vertebrate defensin family of genes comprises two major groups, the α- and β-defensins. The classic defensin motif consists of six cysteines linked by three intramolecular cysteine disulfide bonds, which differ in topology in the α and β groups. α-Defensins are produced mainly in neutrophils and Paneth cells, whereas the β-defensins are widely expressed in epithelial tissues, including those of the respiratory, gastrointestinal, and reproductive systems and the skin. Defensins are synthesized as preproproteins that are subsequently cleaved to generate the active cationic peptides of approximately 3 to 5 kDa [reviewed in Lehrer and Ganz (732)]. It has been suggested that, in the epididymis, a furin-like proprotein convertase is responsible for the cleavage (733). The defensin genes are also clustered on certain chromosomes—chromosome 8 in humans (746,747). A recent study by Zaballos et al. (2004) (741) using the Celera mouse

genome database found exons encoding 23 different β-defensins. Using a combination of RT-PCR and in situ hybridization, these workers found nine of these genes to be highly specific for the epididymis and expressed in distinct epididymal regions.

Members of the β-defensin and β-defensin–like family are expressed in the epididymal epithelium in a region-specific manner and appear to be secreted into the epididymal fluid as demonstrated by variety of techniques, including RT-PCR, in situ hybridization, Western blot analysis, and immunolocalization (726,730,733–739,741–744,748). The androgen regulation of epididymal β-defensins is still unclear. The expression of bin1b in the rat epididymis (730) and HE2 expression in the nonhuman primate epididymis are androgen dependent (726). Although rat β-defensin-1 is androgen regulated in the initial segment and caput regions, this may not be the case in the more distal epididymal regions. To add to the complexity, rat β-defensin-1 expression in the initial segment/caput regions appears to respond slowly to androgen deprivation, suggesting that the action of androgens may be indirect (736). HE2 expression in the human epididymis does not appear to be androgen regulated, but the subcellular distribution of the HE2 variants is androgen regulated (733). Expression of human β-defensin-118 (previously called ESC42) in the nonhuman primate is primarily in the caput region and is androgen regulated. Hence, more studies are warranted to define clearly the androgen regulation of these genes. Although one would suspect that members of the β-defensin family are upregulated during inflammation, it turns out that, at least for rat β-defensin-1, this gene was not upregulated during inflammation of the epididymis induced by lipopolysaccharide from *Pseudomonas aeruginosa* or *Escherichia coli* (736).

The structure–function relationship of the defensins is complex. Outside of the defensin motif, their sequences vary greatly (748a). The antimicrobial activities of human β-defensin-3 (749) and HE2 (733,743) depend very much on both charge and structure. In the case of HE2 expressed in the chimpanzee and the human (726,733), two major isoforms are found in the epididymis, HE2α1 and HE2β1, each related to the β-defensin gene family. HE2β1 clearly has the β-defensin motif and antibacterial activity, yet the processed form of HE2α1 does not have the β-defensin motif but does have antibacterial activity. This suggests that the human epididymis secretes a novel class of antimicrobial peptides (733).

Cathelicidins are another group of antimicrobial peptides that has received attention recently. The human cathelicidin hCAP18 is expressed in the epididymis and associates with prostasomes (exosomes) in seminal fluid and with spermatozoa (727,728,750). Although hCAP18 is processed in neutrophils by proteinase 3 (751), it is processed in seminal fluid by a prostate-derived protease, gastricsin, to generate a 38–amino acid antimicrobial peptide ALL-38. Presumably, this antimicrobial peptide plays an important role in preventing vaginal bacterial infections after intercourse. Cathelicidins have also been identified in other species, for example, rat and mouse CRAMP, and have been shown to have antimicrobial properties (752–754). Members of the family of lipocalins are actively synthesized and secreted by the epididymis of most mammals and have been proposed to play an active role in immune and inflammatory responses (755).

Several of these peptides have functions in addition to their antimicrobial activity. For example, bin1b is potentially involved in the initiation of sperm motility in the epididymis (745), and Eppin (756) may be involved in the ability of spermatozoa to fertilize an egg. It is intriguing that these peptides have evolved to play multiple roles in the epididymis. Future studies should enhance our understanding of these bifunctional peptides and the mechanisms by which they promote sperm maturation and maintain antimicrobial activity.

Lipocalins

Several proteins binding and transporting small hydrophobic ligands and belonging to the lipocalin family have been identified in the epididymis.

Epididymal Retinoic Acid–binding Protein. E-RABP is one of the major epididymal-secreted proteins. It was first identified as one of the four prealbumin proteins migrating after polyacrylamide gel electrophoresis of rat and mouse epididymal cytosol (757) and luminal fluid (88,758), with an apparent molecular weight of 18,500 Da. The mouse and rat proteins were subsequently purified (529,759) and the cDNA isolated (529,760,761). In other studies, two retinoic acid–binding activities were found after separation by ion exchange chromatography of rat and mouse caudal epididymal fluid (762–764). After purification and N-termini sequencing (763,765), these proteins were found to be identical to proteins identified by Brooks et al. (529) and Rankin et al. (764). The predicted amino acid sequence, the ligand-binding specificity, and the x-ray crystal structure showing that the protein has a β-barrel structure and a hydrophobic ligand pocket, indicated that E-RABP belonged to the lipocalin family of small extracellular hydrophobic binding proteins (766,767).

E-RABP is present as a single-copy gene in both the rat and mouse. The gene structures are similar but not identical. The rat E-RABP gene is unusual

compared with other lipocalins in that it has eight exons instead of seven, the more usual number. The cDNA encodes a protein of 188 residues with alternative sites for signal sequence cleavage to give secreted proteins composed of 166 and 169 amino acid residues. In contrast, the murine gene has seven exons, as seen in other lipocalins (767). The cDNA encodes a protein of 185 residues, also with alternative sites for cleavage of the signal sequence to give secreted proteins of 163 and 166 residues. The exon–intron boundaries of the first seven exons are identical for rat and mouse, and the first five exons are quite similar in size to the exons of other members of the lipocalin family. The murine gene is located in the (A3) region of chromosome 2.

A computer analysis of the nucleotide sequence of the flanking regions of the mouse E-RABP gene (*Lcn 5*) showed the presence of 6 new genes, *Lcn 8*, *Lcn 9*, *Lcn 10*, *Lcn 11*, *Lcn 12*, and *Lcn 13*, that evolved by gene duplication. The new genes encode proteins of 175, 178, 182, 170, 193, and 176 amino acids, respectively, with a signal peptide of 16 to 21 amino acids, consistent with these proteins being secreted proteins (768,769). Within this cluster of genes, *Lcn 5*, *Lcn 8*, *Lcn 9*, *Lcn 10*, *Lcn 12*, and *Lcn 13* are specifically expressed in the mouse epididymis. However, each gene has a distinct spatial expression in the epididymis and a different regulation. Lcn 8 and Lcn 9 are expressed in the initial segment only, Lcn 5 is expressed in the distal caput only, whereas Lcn 10 is expressed in the initial segment but also in the upper margin of the distal caput. The *Lcn 8*, *Lcn 9*, and *Lcn 13* genes depend strictly on testicular factors circulating in the luminal fluid. The *Lcn 5* and *Lcn 12* genes depend on androgens circulating in the serum, whereas *Lcn 10* gene expression is regulated both by circulating androgens and by testicular factors supplied from the luminal fluid.

A computational analysis of the human genome identified five putative genes (*E-RABP* [*LCN5*], *LCN8*, *LCN9*, *LCN10*, and *LCN12*) encoding homologous lipocalins. These genes were localized on human chromosome 9q34 (769,770). The genomic organization, chromosomal arrangement, and orientation of these human genes were similar to those of the murine genes.

The cluster of lipocalin genes expressed specifically in the epididymis is localized in an area rich in lipocalin genes on mouse chromosome 2 and human chromosome 9 (771). Interestingly, this epididymis-specific lipocalin cluster is flanked by other members of the lipocalin family, such as prostaglandin D synthase (PGDS) and Lcn 2 (769,771), that are expressed in other tissues besides the epididymis (772,773). This suggests that the epididymis-specific lipocalin cluster may contain a locus control region that enhances lipocalin gene expression only in the epididymis, whereas genes flanking this epididymal gene cluster show expression in other tissues besides the epididymis.

Lipocalin-type Prostaglandin D Synthase. PGDS, formerly identified as β-trace, was first shown to be localized in the central nervous system and secreted in cerebrospinal fluid (774–779). PGDS is also localized in the male genital tract, where it constitutes one of the major secreted epididymal proteins in rodents, domestic animals, and humans [mouse (780–782), rat (772,782,783), hamster (781), ram, stallion (784,785), bull (786), monkey (787), and man (788)]. The cDNA for PGDS was first isolated from a rat brain library (789), and subsequently from many species, including humans and zebrafish (784,786,790–794). The cDNA for PGDS encodes a protein of 180 to 190 amino acid residues with a signal peptide of approximately 20 amino acids removed in the mature protein and 2 N-glycosylation sites. The tertiary structure of the rat and mouse recombinant proteins identified PGDS as a lipocalin (787,795). The gene organization is similar to that of other lipocalins in terms of number and size of exons and phase of splicing introns (796–798).

In the epididymis, PGDS distribution varies among species. In the mouse, PGDS mRNA is not detected in the initial segment, but is expressed in all other regions of the epididymis (781), whereas in the rat (772,799), ram, stallion (784), and bull (800), there is a higher expression of PGDS mRNA in the caput than in the cauda epididymidis. In rodents, the secreted protein accumulates in the caudal fluid of mouse, rat, and hamster (781,786), whereas in ram and stallion the highest concentration of the protein is found in the fluid of the proximal epididymis (784).

PGDS expression is regulated by androgens; it increases in parallel with endogenous androgen levels during sexual maturation, decreases after castration, and is restored with exogenous androgens (772,782, 784,799).

Lipocalin 2. Lipocalin 2 (Lcn 2), also known as NGAL (neutrophil gelatinase-associated lipocalin precursor), uterocalin, *neu*-related lipocalin, and 24p3, was originally identified as a component of neutrophil granules, but is also expressed in epithelia in response to inflammatory signals (801). It is present in epididymal fluid, although it is not one of its major components. This protein is expressed in various organs but, in the male reproductive tract, is restricted to the epididymis (773). Lcn 2 is a 25-kDa protein originally purified from human neutrophils (802). It is a 178–amino acid protein with glycosylation sites and a 20–amino acid signal peptide (803–806). X-ray crystallography revealed that the protein has the typical lipocalin structure, but the calyx is shallower and broader than in other lipocalins (807).

In the epididymis, Lcn 2 is expressed in the caput (773) and is under androgen control (769); the protein accumulates in the caudal fluid (773). Unlike E-RABP, sequential washing of spermatozoa with low salt and high salt left a substantial amount of Lcn2 immunoreactivity in the Triton X-100 and the sodium dodecyl sulfate (SDS) sperm extract, suggesting that Lcn 2 is bound to spermatozoa (773).

Lipocalin Function. Lipocalins comprise an ancient family of proteins widely distributed among species, with the phylogenetic tree rooted in bacteria (808). L-PGDS gene is found in fish, amphibians, and many mammalian species (787), and is believed to be the ancestral gene of the epididymal lipocalin cluster (769), whereas the E-RABP gene is found in reptiles (809) and mammals, including humans (769). Genes preserved in different lineages are likely to possess important biological functions that are conserved from reptiles to primates. In addition, gene duplication provides genetic redundancy and improves the fitness of the organism (810). However, gene duplication, through neofunctionalization or subfunctionalization, also allows adaptation and functional innovation (811,812). Species comparison, even over short evolutionary distances, reveals dramatic expansions and contractions of gene families (813–817). It is all the more remarkable that the organization of the lipocalin gene cluster is conserved in mouse and human, with similar gene number, order, and orientation (769). This implies that functional constraints have maintained the integrity of the cluster since the last common ancestor of the two species, 75 million years ago.

The basic function of lipocalins, determined by x-ray crystallography, is to bind small hydrophobic ligands. The folding of the protein into a barrel structure with a deep calyx is well suited for this function. As extracellular secreted proteins, lipocalins function as carriers for these ligands, and their biological properties depend on their respective ligand. However, endogenous ligands are known for few lipocalins because the methods of isolation used disrupt the complexes. The endogenous ligands of LCN 1 (human tear lipocalin) belong to a class of lipophilic molecules such as fatty acids, phospholipids, glycolipids, and cholesterols (818). By scavenging these molecules, LCN 1 may act as a general protection factor for epithelial surfaces. In vitro, LCN 1 also binds retinol (819). In addition, LCN 1 binds to microbial siderophores with high affinities and effectively inhibits bacterial and fungal growth under iron-limiting conditions. Therefore, as discussed previously, LCN 1 appears to be part of the complex defense system involved in protection against harmful molecules and microbial and fungal infections (820). LCN 2, the only lipocalin with a known endogenous ligand, is part of the lipocalin cluster on human chromosome 9. Its ligand is a breakdown product of enterobactin, a siderophore that is used by bacteria for uptake of the essential nutrient iron (821). This is consistent with the potent antimicrobial action of LCN 2 (822). LCN 2 is also upregulated in response to inflammatory stimuli (823,824). This suggests that LCN 2 may have a role in the innate immune response to bacterial challenges in the epididymis. Mouse Lcn 2 has also been reported to deliver ferric iron to spermatozoa through internalization (825), and to enhance sperm motility through an elevation in intracellular pH and increased intracellular cAMP accumulation (826). Lcn 2 may have a dual function in the epididymis.

Ligand affinity of the other lipocalins has been determined in vitro. L-PGDS binds all-*trans* or 9-*cis* retinoic acid and all-*trans* or 13-*cis* retinaldehyde, but not all-*trans* retinol (827). It also binds thyroid hormone, biliverdin, and bilirubin (828). However, similar to bin1b and Eppin, discussed earlier, PGDS is a dual-function protein. In the cell, it acts as an enzyme producing PGD_2, a potent endogenous somnogen and pain modulator as well as anti-inflammatory agent; after secretion, it functions as a lipophilic ligand carrier. This dual function may reflect subfunctionalization in the lipocalin cluster. After inactivation of the PGDS gene, the main abnormalities that occur are in the regulation of sleep and pain responses, but no obvious phenotype was reported in the male genital tract, reflecting perhaps a built-in redundancy in the lipocalin cluster (829). Nevertheless, PGDS has been reported to be a biochemical marker of sperm quality in human semen (830) and to be correlated with fertility in bulls (786). Another study failed to find the correlation statistically significant, although the lowest PGDS contents were found in rams and bulls with the lowest fertility and the highest content in animals with normal or high fertility (781). This suggests that PGDS affects male fertility, but its role is either not essential or can be compensated by other lipocalins in the cluster.

It is likely that retinoic acid is the endogenous ligand for E-RABP, which binds in vitro all-*trans* and 9-*cis* retinoic acid, but not retinol. E-RABP inactivation produced a phenotype similar to that seen in transgenic mice expressing a dominant negative mutation of RARα, albeit with a lower severity and penetrance [K. Suzuki, unpublished observations, cited in Costa et al. (606)]. The transgenic mice expressing the dominant negative RARα were either infertile or had reduced fertility. The epithelium of the cauda epididymidis had undergone squamous metaplasia with tubule rupture, sperm leakage into the connective tissue, and inflammatory reaction. This indicates that interaction of retinoic acid with its receptor is essential for the maintenance of the epithelium of the

cauda epididymidis. These observations are consistent with E-RABP functioning as an intraluminal carrier protein delivering retinoic acid to the epithelium of the cauda, and the fact that the epithelium of the cauda epididymidis is most affected in vitamin A deficiency (831).

In summary, gene duplication of a PGDS-like lipocalin gave rise to a new epididymal lipocalin family as amniotes appeared during evolution. Internal fertilization is a common feature of amniotes and this process leads to complex and changing environmental conditions for male gametes. Gene duplication, which led to an epididymal lipocalin gene cluster, may have allowed an early adaptive response to a new environment; its conservation through 250 million years strongly suggests that the epididymal lipocalins have important functions.

Lipocalins on mouse chromosome 2 and human chromosome 9 are involved in immunoregulatory, anti-inflammatory, and antibacterial responses (755). As discussed previously, the epididymis has a complex host defense system involving, in particular, the defensins. It is likely that the lipocalins of the epididymal cluster are part of this network, either through their antimicrobial action, as with Lcn 2, or by preserving the integrity of the luminal compartment of the epididymis, as with E-RABP (Lcn 5).

AGING

Effects of Aging on the Appearance of the Epididymal Epithelium

As discussed previously, numerous studies have focused on the structure, function, and regulation of the epididymis during prenatal and postnatal development and in the adult, yet very few studies have investigated the effects of senescence on epididymal structure and function. Less than 20 publications have either directly or indirectly addressed this question. In the aging rabbit, Cran and Jones (1980) (832) showed that there was an absence of spermatozoa in the epididymal lumen, an accumulation of lipofuscin pigmented bodies in principal and basal cells, cytoplasmic vacuoles in the distal segments, and intranuclear inclusions. In the aging cat epididymis, Elcock and Scoeming (833) observed occasional intraepithelial cysts and hyperplasia of the epithelium, as well as eosinophilic to amphophilic round cytoplasmic bodies in interstitial cells, and proteinaceous luminal debris. In the hamster, Calvo et al. (833a) found that the cauda epididymidis was particularly affected by aging. Aged hamsters presented involutive changes in the epididymis, including a decrease in tubular diameter in the cauda epididymidis. Principal cell ultrastructure showed the appearance of damaged mitochondria and bundles of filaments. The presence of large, electron-dense vacuoles in some clear cells was unusual. Decreases in sperm concentration and an apparent decrease in motility were noted also. Decreased epididymal sperm counts, brown patches or lipofuscin, arteriosclerosis, lymphocyte infiltration, and the emergence of sperm autoantibodies have been reported in other studies [rat (834,835), human (836,837), monkey (838)].

Results from many of these studies are difficult to interpret owing to the difficulty in establishing whether the age-dependent effects observed are due to aging of the tissue per se or to other conditions, such as arthroscleroses or carcinoma in other tissues impinging on the epididymis. The brown Norway rat has been established as a valuable model for the study of the aging male reproductive system (90,839–842). This strain of rat has a long life span, does not exhibit many of the age-related pathologies found in other rat strains, such as pituitary and Leydig cell tumors, does not become obese, and is the first rat strain to have had its genome sequenced (839,840,843); similarly, in humans, aging in men leads to dramatic changes in the seminiferous epithelium and to decreases in spermatogenesis and steroidogenesis [reviewed in Neaves et al. (844), Johnson et al. (845), Zirkin and Chen (846), and Viger and Robaire (847)], with no decrease in the concentrations of gonadotropins.

Using the brown Norway rat as a model to study aging of the epididymis, several clear observations have emerged (96,119,136). Although the luminal diameter and epithelial height were not affected by age, there was a marked progressive increase in the thickness of the basement membrane during aging. In young rats, the relative contribution of clear cells increased in the distal segments, resulting in a relative decrease in the numbers of principal cells, whereas halo cells were rare. With increasing age, there was a decrease in the proportion of principal cells and basal cells in all segments of the epididymis, of narrow cells in the initial segment, and of clear cells in the corpus epididymidis. This decrease was accompanied by a proportional striking increase in the number of halo cells in each segment (96).

Several dramatic changes occurred along the epididymis in specific cellular structures during aging (96). In the initial segment, basal cells emitted an extensive network of pseudopods extending downward toward the basement membrane, and the number and size of halo cells increased progressively during aging. In the caput epididymidis, principal and basal cells did not appear to change, whereas clear cells changed dramatically: they appeared swollen and bulged into the lumen; their nuclei were irregularly shaped and displaced to the upper half of the cell; lysosomes, enlarged and dense, were often fused

with lipid droplets; and the presence of lipofuscin became evident. In the corpus epididymidis, a remarkable increase in the size and number of lysosomes was seen in principal cells. The emergence of a localized region with large vacuoles reflected the major effect of age in the proximal distal corpus/proximal cauda epididymidis. Although some principal cells in old animals had a normal morphology, many others contained extremely large vacuoles. Endosomes and lysosomes often appeared to be emptying their contents into the large vacuoles, and debris from spermatozoa was found in giant vacuoles. Serial sections of these vacuoles revealed that entire spermatozoa were found in the vacuoles, thus leading to the hypothesis that mechanisms controlling intracellular trafficking are impaired as animals age (96).

There was a dramatic increase with increasing age in the number of halo cells (composed of monocytes, helper T lymphocytes, and cytotoxic T lymphocytes) throughout the epididymis in a segment-specific manner (119). There was a segment-specific recruitment of cytotoxic T lymphocytes and monocytes–macrophages in the epididymal epithelium of aged rats whose epididymal lumen contained few spermatozoa. Thus, accumulation of damaged epithelial cells and antigens of germ cell origin, leaking through a dysfunctional blood–epididymis barrier, may contribute to the active recruitment of immune cells with age (136). This age-dependent increase in the number of halo cells in the epididymis, in conjunction with the fact that the blood–epididymis barrier should protect from immunological attack, led to investigation of whether there are changes in the structure and function of this barrier with age in the brown Norway rat model (136). Based on changes in the distribution of several immunocytochemical markers of this barrier (occludin, zonula occludens-1, and E-cadherin), as well as in lanthanum nitrate permeability, a clear breakdown of the barrier during aging was shown to occur selectively in the corpus epididymidis.

Molecular and Functional Effects of Aging in the Epididymis

Using both gene expression profiling (cDNA microarrays) and Northern blot analysis, segment-specific changes have been found to occur along the epididymis during aging (464,847). The overwhelming effect was a decrease in gene expression during aging. In the initial segment, corpus, and cauda epididymidis, more genes decreased in expression with age than did not change. Interestingly, the magnitude of the decreases in expression was considerably larger in the corpus and cauda epididymidis, where expression of 83% (211 of 254) and 62% (157 of 254), respectively, of the genes decreased by greater than 50% with age. This is in contrast to the initial segment, in which only 31% of the genes (78 of 254) expressed decreased by at least 50%; the caput epididymidis was the only segment in which the expression of a large proportion of the genes did not change with age (less than 33% changed between young and old). No genes had increased expression with age in any of the four segments of the tissue; however, the expression of four transcripts was increased in a segment-specific manner (464).

Changes in the expression of specific genes/gene families are of particular interest. Expression of the mRNAs for 5α-reductases (particularly the type 1 isozyme) was among the first to decline during aging, whereas the message for the androgen receptor was not affected (847). Changes in the expression of components of intracellular degradation pathways (i.e., proteasome components) were most dramatic in the distal epididymis. Although in the initial segment and caput epididymidis, either no change or small decreases (<33%) were seen with age, in the corpus and cauda epididymidis essentially all proteasome components showed remarkable decreases in intensity of expression (>67%). Cathepsins, a family of cysteine proteases implicated in proteolytic processes, are abundantly expressed in the epididymis (353,474,847). Of the six cathepsin transcripts detected in the epididymis, the relative intensity of cathepsin K was affected by age to the greatest extent in the initial segment, whereas that of cathepsin H and B decreased most in the more distal segments of the epididymis; cathepsin E transcripts decreased to below the level of detection in all regions of aged epididymides. Oxidative stress–related genes, such as copper-zinc superoxide dismutase, showed a small decrease with age in the initial segment, but a very large decrease in the corpus epididymidis. The relative intensity of expression of glutathione synthetase decreased dramatically with age in the initial segment, corpus and cauda epididymides, whereas epididymal secretory glutathione peroxidase (GPX5) showed the largest decrease in relative intensity in the initial segment and GPX4 showed the largest decrease in relative intensity in the cauda epididymidis (464). The changes seen with age for many of these gene transcripts were consistent with what has been observed by others on the effects of aging in various tissues in a wide spectrum of animals, from flies (848) to mice [muscle (849); brain (850)].

Mechanisms Underlying Aging of the Epididymis

Numerous hypotheses have been put forward regarding the underlying cause or causes of aging. These include the specific gene theory (851), such as

the *klotho* gene (852), the telomere theory (853), altered gene expression due either to mutations (854) or altered DNA methylation status (855), the network or immune theory of aging (856), and the free radical (oxidative stress) theory (857). However, there is no consensus regarding any one hypothesis, and too few studies have been done on the epididymis to demonstrate the primary role of any one or combination of these mechanisms. There is a significant recruitment of immune cells into the epididymis as animals age, and this recruitment is greatest in parts of the tissue exhibiting greatest damage (119,136). Although it is possible that this immune response is a cause of the epithelial damage, it is perhaps more likely that the damaged tissue and ruptured blood– epididymis barrier are the attractants of immune cells. It was also shown that the amount of apoptosis seen in epididymides was higher in aged than in young mice, and that this increased rate of apoptosis could be prevented by the administration of testosterone (858).

There are several lines of suggestive evidence that point to the association of oxidative stress with changes induced by aging in the epididymis. These include region-specific accumulation of lipofuscin (859), changes in the distribution of glutathione S-transferases (860), formation of intracellular vacuoles (96), and altered expression of genes associated with oxidative stress (464). Therefore, it is reasonable to speculate that oxidative stress is a key factor in mediating the dramatic response of the epididymis to age. However, little is known about potential changes with age in the ability of the epididymis to respond to stress or altered endocrine environments.

In addition, two issues have been addressed with respect to the effects of advancing age on epididymal functions: the first relates to tissue weight, sperm number, and the effect of sexual experience on sperm number in this tissue (861), and the second to the effects of testosterone administration on sperm number and sperm maturation status ("sperm profile") (40). Sexual experience in rats was shown to lead to an increase in epididymal sperm counts, which peaked at 9 months but returned to within 5% of that at 3 months when animals were 24 months old. Only the weight of the epididymides increased between 3 and 24 months, reaching a value at 24 months that was 28% greater than that of the 3-month-old animals. However, sexually inexperienced rats had a 31% decrease in epididymal spermatozoa between the ages of 3 and 24 months, although epididymal weight still increased by 25% (861). The lack of correlation between epididymal weight and epididymal sperm content is completely unexpected and provides an important clue to potential histological changes taking place in the tissue. The administration of exogenous testosterone could not prevent the loss in sperm number, but did prevent the changes seen in sperm profile, suggesting that the epididymis of aged animals retains its ability to respond to endocrine manipulations (834).

Changes in Spermatozoa during Aging

Several clinical studies, as well as studies using models of animal aging, indicate that significant changes occur in spermatozoa as males enter advanced age and that such changes have consequences for progeny (862–866). Determining whether these changes are due to alterations taking place in the testis or the epididymis is challenging. The existence of a paternal age effect on semen parameters, fertility, and anomalies in children demonstrates that the quality of spermatozoa and the genetic integrity of otherwise apparently healthy spermatozoa are not immune to the effects of time.

Although genetic changes in spermatozoa during aging, such as nondysjunction and autosomal dominant disorders that exhibit paternal age effects [e.g., Down's (867) and Klinefelter's syndromes (868) and achondroplasia (869)] are clearly of testicular origin, others, such as changes in the DNA methylation pattern (epigenetic changes) (870), altered sperm motility, and retention of cytoplasmic droplets, are more likely to have an origin in the epididymis. Using the brown Norway rat as an model for male reproductive aging, it was shown that there is a marked increase with age in the number of abnormal flagellar midpieces of spermatozoa, that the percentage of motile spermatozoa was significantly decreased in the cauda epididymidis of old rats, and that the proportion of spermatozoa that retained their cytoplasmic droplet was markedly elevated (871). The origin of these changes may still be ascribable to altered proteins made during spermatogenesis, but the possibility that altered epididymal epithelial functions contribute to such changes must be considered.

Another mechanism that may allow for sperm quality control has been observed to act at the level of the epididymis and involves the cell surface ubiquitination and subsequent phagocytosis of abnormal spermatozoa (306). However, large-scale phagocytosis of spermatozoa by the epididymal epithelium is not observed in young or old animals (307). To date, it has not been clearly demonstrated that genetic damage–specific apoptosis or phagocytosis does occur or that there is an age-dependent increase in this process.

The multiple effects of aging on progeny outcome, sperm morphology, the acquisition of motility, and the shedding of the cytoplasmic droplet

all indicate that the quality of spermatozoa is affected by aging.

THE EPIDIDYMIS AS TARGET FOR XENOBIOTICS

Xenobiotics that have been shown to act on the epididymis can be broadly divided into two major, somewhat overlapping, categories. The first consists of those chemicals for which the epididymis is the explicit target; a number of compounds that have the potential to act as part of a male contraceptive formulation fall into this group. The second is made up of therapeutic agents or known toxicants that are targeted principally at other organs but also act, as a side effect, on the epididymis. For some of these compounds, their effects on the epididymis are at the level of the epididymal epithelium; for others, effects have been demonstrated on sperm traversing the epididymis; and for others still the site of action is mixed or not resolved. Various aspects of the action of drugs on the epididymis have been thoroughly reviewed (872–877).

Demonstrating the direct action of chemicals on the epididymis is challenging because if, subsequent to administration or exposure to a chemical, there is an effect on the epididymal epithelium or on epididymal spermatozoa, it becomes essential to establish that such an effect is not mediated at the level of the hypothalamus, pituitary, or testis. Furthermore, because epididymal histopathological study and sperm function tests have not been standard in drug development and the assessment of toxicants, the action of xenobiotics on this tissue may often have been overlooked.

Chemicals for Which the Epididymis is an Explicit Target

Selective modification of functions carried out either in or by the epididymis should be of major value not only in the development of male contraceptives, but for the treatment of male fertility problems with an etiology that arises in the epididymis. Because spermatozoa reside a relatively short time in the epididymis and because no genetic transformation is known to occur as spermatozoa undergo epididymal maturation, the epididymis can be viewed as an ideal target for the development of male contraceptives or drugs that can enhance the maturation process. Although no therapeutic agent has yet been developed that meets these objectives, a large number of approaches have been pursued to attain these goals. Most of these agents can be grouped into drugs that act by modulating hormonal dependence, neuronal activity, or metabolism.

Hormonal Regulation

Although, as described previously, many hormones and hormone receptors have been found in the epididymis in various species, we focus on androgens and estrogens, hormones for which the consequences of manipulating the endocrine signal have been reported.

Blocking Androgen Action. Near the beginning of the 20th century it was established that the epididymis is highly dependent on androgens (12). Several laboratories have determined the effects on the epididymis of agents that act as androgen receptor antagonists, such as cyproterone acetate or hydroxyflutamide (878,879). These compounds successfully block epididymal functions and cause a reduction in tissue weight. Interestingly, unlike the case with other androgen-dependent tissues, such as the prostate or seminal vesicles, exposure to androgen receptor antagonists does not result in changes in intracellular androgen receptor localization in the epididymis (880). The therapeutic potential of these compounds as male contraceptives targeting epididymal functions is limited, however, because, as expected, the action of androgens in other target tissues was also blocked, producing side effects equivalent to a chemical orchidectomy.

Blocking the Formation of Dihydrotestosterone. Testosterone is normally not synthesized de novo in the epididymis but is converted to the more biologically active androgen, DHT, by steroid 5α-reductase (517). There are two isozymes of 5α-reductase (types 1 and 2), both of which are abundantly expressed in the epididymis (523,881). It has been established that DHT is essential for the maturation of spermatozoa and that the androgen found in the nuclei of epididymal cells is DHT (476,882). Thus, one approach to block androgen action in the epididymis is to inhibit DHT formation. Several compounds have been reported to inhibit either one or both forms of 5α-reductase in many tissues, including the epididymis (883–889). The first report was of a family of 5,10-secosteroids that are suicide substrates (irreversible inhibitors) of the enzyme in vitro and have been shown to inhibit the epididymal enzyme (884). In a series of studies, Rasmusson's group (885,890) found that 4-aza steroids were effective inhibitors of steroid-5α-reductase in the prostate and a variety of other tissues. The lead compound (diethyl-4-methyl-3-oxo-4-aza-5α-androstane-17 β-carboxamide [4-MA]), a competitive inhibitor of the epididymal enzyme (886), ushered in a new era

in potent inhibitors of this enzyme activity; this inhibitor was shown in one study to alter the fertility of male rats (891). The first commercially available inhibitor of 5α-reductase, finasteride, also a member of the azasteroid family, has a predominant effect on the type 2 isozyme (892), whereas more recently developed agents, such as dutasteride or PNU157706, act as dual inhibitors (887,893). Using PNU157706 as a model inhibitor, it was shown that treatment of adult rats with this agent results in pronounced effects on the expression of genes involved in signal transduction, fatty acid and lipid metabolism, regulation of ion and fluid transport, luminal acidification, oxidative defense, and protein processing and degradation, all processes essential to the formation of the optimal luminal microenvironment required for proper sperm maturation (889). Treatment of adult rats with this dual inhibitor had effects on both sperm quality and pregnancy outcome but did not result in an arrest of fertility (894).

Blocking Estrogen Action. Although the presence and selective localization of both ERα and ERβ along the epididymis has now been established in several species (485,567,568), few studies have focused on elaborating the consequences of blocking estrogen action in the epididymis. It is clear that the removal of estrogen action (null mutations) in the efferent ducts resulted in a dramatic reduction in the fluid uptake capacity of that tissue and consequent infertility (571), but less is known about the consequences of withdrawing estrogen from the epididymis. Some tantalizing lines of evidence are that selective blockade of estrogen action, whether by treating with an aromatase inhibitor or an ER antagonist, resulted in blocking the oxytocin receptor in the rabbit epididymis (175), and that by using an ERα blocker, ICI182,780, the apical cytoplasm of narrow and clear cells was affected (895).

Neuronal Regulation

An alternate means by which xenobiotics can affect epididymal functions is by increasing or decreasing sperm transit time. Either administration of guanethidine, a drug that causes chemical sympathetectomy, or surgical sympathetectomy resulted in a marked increase in sperm transit time as well as in cauda epididymal weight without other apparent effects (896,896a,897). In contrast, several drugs accelerate transport of spermatozoa through the epididymis. In 1975, Meistrich et al. (166) demonstrated that treatment of mice with estradiol resulted in an increased rate of transport of sperm through the epididymis. Administration of chloroethylmethanesulfonate, a chemical that causes a reduction in serum testosterone, or hydroxyflutamide also led to accelerated sperm transit (897). Sulfapyridine, a metabolite of the antibiotic sulfasalazine, caused a marked reduction in fertility in both rat and human; although its mechanism of action has yet to be elucidated, it was also found to accelerate sperm transit through the epididymis (898).

Metabolism

Several compounds that act on enzymes in the cellular metabolic pathways have been tested as potential male contraceptives. The ones that hold the greatest potential are reviewed in the following.

α-Chlorohydrin. In the late 1960s, a series of studies appeared on the ability of α-chlorohydrin, a monochloro derivative of glycerol, to inhibit fertility reversibly in an array of species (899,900). This drug is converted by glycerol dehydrogenase to 3-phosphoglyceraldehyde, an inhibitory analog of the substrate for glyceraldehyde-3-phosphate dehydrogenase (GADPH), and acts, therefore, at low doses to inhibit this key metabolic enzyme in spermatozoa residing in the cauda epididymidis (901) and reduces sperm motility (902). At higher doses, however, α-chlorohydrin caused the formation of spermatoceles in the caput epididymidis of rodents, resulting in the blockade of sperm transport through the tissue and the consequent atrophy of the seminiferous tubules due to increases in back pressure (903). Wong and colleagues (1977) (904) demonstrated that α-chlorohydrin blocked the resorption of sodium and water from the cauda epididymidis. Although α-chlorohydrin inhibits a ubiquitous metabolic enzyme, its selectivity of action on spermatozoa is based on the fact that spermatozoa have a GADPH isoenzyme with a higher molecular weight that is not cytosolic (905). Nevertheless, severe toxicity (bone marrow depression, kidney, liver) was associated with this compound when it was tested in primates (900,906), and therefore it will not be developed as a contraceptive in humans.

6-Chloro-6-Deoxy-Glucose. In 1978, Ford and Waites (907) described another antifertility compound, 6-chloro-6-deoxy-glucose, with the ability to inactivate spermatozoa from the cauda epididymides; it also acts by blocking GADPH (903). This compound was also found to affect different functions of the epididymis, but because of its neurotoxicity in primates (associated with its effects on glucose transport and inhibition of GADPH), it is not likely to be accepted as a male contraceptive in humans.

Gossypol. Although gossypol, a chemical found in cotton seed oil, has been studied extensively as a potential male contraceptive agent (908,909), its mechanism of action remains poorly understood. Although it has been shown to have a high contraceptive efficacy, its toxicity, probably mediated through a hypokalemic

action, is likely to preclude the clinical use of gossypol as a "stand-alone" contraceptive (910); it may have a role at low does in combination with steroidal contraception (911). Gossypol caused shedding of spermatozoa from the seminiferous tubules, separation of sperm heads from tails, and a decrease in sperm number in the cauda epididymides (908). Studies by Romualdo et al. (2002) (912) have also shown that treatment of rats during the pubertal time window resulted in an increase in the appearance of round cells in the epididymal lumen; these cells were most likely of epididymal epithelial origin because they stained for cysteine-rich secretory protein (CRISP)-1, also known as protein E, a marker of epididymal principal cells. CRISP-1 staining was not found in testicular cells, providing further evidence that the epididymis is indeed a target organ for gossypol (912).

Chemicals Targeted Principally at Other Organs that Also Act on the Epididymis

The epididymis has not usually been studied as one of the target tissues for the toxicological effects of drugs or toxicants, and consequently little is known about the importance of this tissue as a target for the toxic effects of xenobiotics. However, there are a few instances where the epididymis has been demonstrated to be a target of drug action.

Cyclophosphamide

Cyclophosphamide, a widely used anticancer and immunosuppressive drug, has been shown to act as a male-mediated developmental toxicant [reviewed in Robaire and Hales (913)]. Administration of this drug resulted in an increase in the number of clear cells and in the number of spermatozoa with abnormal tails in the epididymis of rats (80). By using several complementary approaches, Qiu et al. (1992) (145) demonstrated that the increase in postimplantation embryo loss observed among progeny sired by male rats subjected to short-term (4 or 7 days) treatment with cyclophosphamide was mediated by the action of this drug on spermatozoa while transiting through the epididymis. Whether the drug action was mediated by an effect on the epididymal epithelium or acted directly on spermatozoa is unresolved.

Fungicides

Several fungicides, including mancozeb, ornidazole, carbendazim, and benzimidazole, have effects on male fertility (914–917). For some of these compounds, sufficiently detailed studies have been undertaken to indicate that there is a selective action at the level of the epididymis. For example, benzimidazole causes occlusion of the epididymis and the efferent ducts, and ornidazole causes a decrease in sperm motility. Whether the members of this family of drugs have a similar mechanism of action is not clear, but treatment with ornidazole caused an inhibition of GADPH, a similar action to that of 6-chloro,6-deoxy fructose and α-chlorohydrin (918).

Methyl Chloride

Another mechanism by which a xenobiotic can affect the epididymis is by increasing the inflammatory response. Exposure of male rats to methyl chloride, an industrial gas, resulted in infertility (a high rate of dominant lethal mutations) that was associated with testicular degeneration, epididymal inflammation, and sperm granuloma formation. Chellman et al. (1986) (919) demonstrated that the infertility was associated with epididymal inflammation and that treatment with an anti-inflammatory drug resulted in a reversal of these effects.

Sulfonates

Exposure to either ethyldimethylsulfonate or chloroethylmethanesulfonate resulted in what is commonly viewed as a selective destruction of Leydig cells and commensurate reduction in testosterone production (920). These chemicals also caused a reduction in fertility. Although the effect on fertility may be ascribed to the reduction in testosterone, Klinefelter et al. (1994) (921) clearly demonstrated that these chemicals have, at similar doses, a direct effect on the epididymis, independent of their action on Leydig cells. The height of epithelial cells along the corpus and proximal cauda epididymidis was reduced, there was a reduction in the fertilizing ability of cauda epididymal spermatozoa, and these spermatozoa had a reduction in protein content. Although the specific mechanism of action of this family of chemicals on the epididymis is still unresolved, a selective reduction in SP22, a protein that is specifically acquired by spermatozoa as they traverse the epididymis, has been reported (922).

In addition to the effects of xenobiotics seen after the exposure of adults, a number of studies have demonstrated that exposure during fetal, early postnatal, or pubertal time windows can result in abnormalities in the epididymis (weight, histology) or in the spermatozoa (number, motility) present in this tissue. Some of these chemicals include dibutyl phthalate, a plasticizer (923,924), 2,3,7,8-tetrachlorodibenzo-rho-dioxin (925), and pesticides such as PCB 169 and methoxychlor (926).

PERSPECTIVE AND FUTURE DIRECTIONS

Since the early 1990s, we have witnessed an explosion of information on a wide range of facets of epididymal gene and protein expression and their regulation, effects of null mutation and targeted transgenic expression of specific molecules, secretion of proteins by epididymal epithelial cells and their interactions with spermatozoa, and mechanisms for creating an ever-changing luminal microenvironment in the fluid surrounding spermatozoa in the duct lumen. Many of these advances have been attained thanks to the application of novel technologies that range from laser capture microscopy to gene expression profiling by microarrays, from in situ electroporation to tissue-targeted gene deletions and the development of immortalized cell lines.

We are beginning to grasp the remarkable complexity of the epithelium lining the epididymal duct and the milieu it creates in the lumen that allows spermatozoa to mature and be stored and protected. However, in spite of these great strides, some of the major questions relating to the epididymis remain unanswered. What mechanisms regulate the lobe-/segment-/region-specific expression of genes and proteins along the epididymis? How can adjacent principal cells have such dramatically different levels of expression of various proteins? What are the actual functions of each of the highly differentiated cell types in the duct? Why have we not yet succeeded in isolating and immortalizing each of these cell types? Is there a specific protein secreted by the epididymis that allows spermatozoa to attain a mature state, or is there a series of multiple, inter-related steps involving changes in proteins, sugars, and lipids? What aspects of the cauda epididymal fluid allow sperm to remain dormant and functional for protracted periods? What is the role of the assortment of antimicrobials being discovered in this tissue? Why is primary cancer of the epididymis so rare? These and other questions have been challenging reproductive biologists for many decades. With the plethora of new tools that have been developed and that will certainly continue to emerge, we are now poised to elucidate the working of this remarkable tissue.

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