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Morphologic Changes in Efferent Ductules and Epididymis in Estrogen Receptor-α Knockout Mice

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ABSTRACT: Estrogen has been shown to have an important role in fluid reabsorption in efferent ductules of the testis. Our previous study of the estrogen receptor-α knockout mouse (ERKO) showed that the efferent ductules and rete testis were primary targets of estrogen receptor function. In the present study, a more comprehensive evaluation of the ERKO male reproductive tract was performed to determine the severity of effects in efferent ductules as well as the epididymis. The following observations were found in ERKO males: 1) blind-ending efferent ductules were more prevalent in ERKO than in wild type (WT) tissues; 2) glycogen-containing cells were observed at the rete testis–efferent ductule junction; 3) the tubular diameters of the efferent ductules and initial segment epididymides were dilated; 4) efferent ductules were dilated between 130 to 300% over wild type ductules; 5) efferent ductule epithelial height was reduced nearly 50%; 6) microvilli of nonciliated cells of efferent ductules were 64% shorter in length; 7) cilia were reduced in number; 8) initial segment epithelium was displaced into regions adjacent to the rete testis and in short segments of the common region of efferent ductule; 9) apical, narrow, and clear cells of the epididymis also were abnormal in some regions; 10) in the corpus and cauda regions, sperm granulomas were noted in one third of the ERKO males. In conclusion, the entire reproductive tract is affected in ERKO males. The cells showing the greatest effects were estrogen-receptor–positive cells. It appears that in the ERKO mouse there are developmental anomalies that must be considered separately from adult dysfunctional changes in the male reproductive tract.

Key words: Testis, histopathology.

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Estrogen appears to be more important in male reproduction than was first hypothesized. Estrogen receptors (ER) are abundant in the male reproductive tract (Fisher et al, 1997; Goyal et al, 1997; Hess et al, 1997b; Schleicher et al, 1984), but ERα and ERβ are expressed differentially, and only ERα appears to retain a dominant role in the testis and epididymis (Couse et al, 1997; Kuiper et al, 1997; Kuiper et al, 1996; Saunders et al, 1997). ERβ appears to be more important in the accessory organs (Kuiper et al, 1997). The importance of estrogen in male reproduction was first recognized when the ERKO mouse was found to be infertile (Eddy et al, 1996; Lubahn et al, 1993). Although spermatogenesis in ER-α knockout mouse (ERKO) males appeared to be normal during early development, as the males aged, sperm production declined, and epididymal sperm appeared abnormal (Eddy et al, 1996). It also was noted in ERKO males that the seminiferous tubule lumen became dilated over time (Hess et al, 1997a). However, the pathophysiologic or biochemical mechanisms responsible for these changes in testicular function and male infertility were not known.

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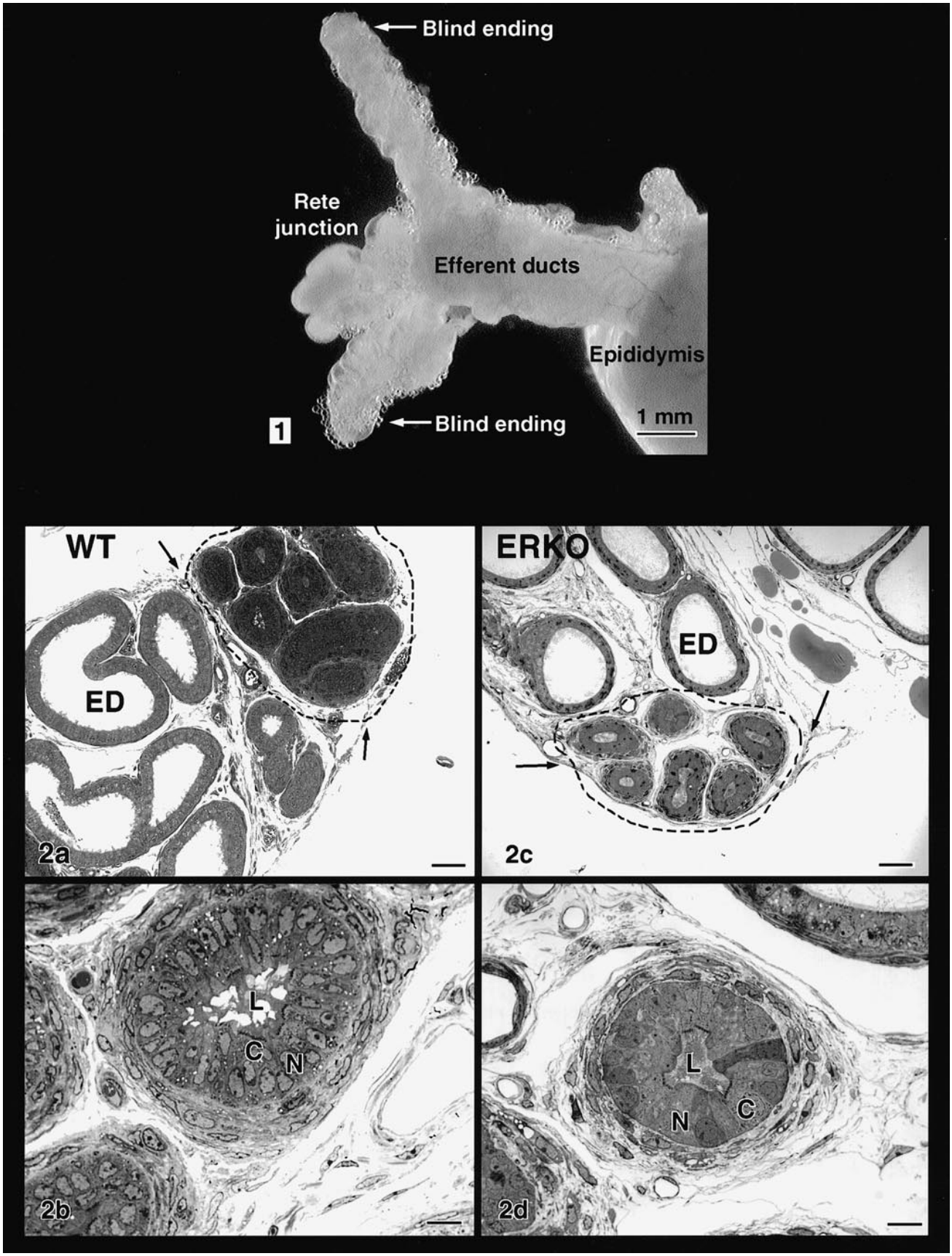
To investigate these mechanisms, our laboratory examined the ERKO male for histopathologic changes in the excurrent ducts of the testis. We found a major lesion in efferent ductules of the testis, a site known for its responsibility in reabsorbing nearly 90% of the luminal fluids (Clulow et al, 1994). In ERKO males, the rete testis was swollen severalfold, and efferent ductules were dilated, suggesting that fluid was not being reabsorbed but was accumulating in the testis (Hess et al, 1997a). Basic histologic methods showed a reduction in epithelial height and a loss of endocytotic vesicles and lysosomal structures in these ductules of ERα deficient males, providing further indication of a decrease in fluid reabsorption. Most importantly, an examination of testicular weight over time revealed that ERKO testes nearly doubled in mass around 70 to 80 days of age, prior to a steady decline in weight over the next 100 days, leading to total atrophy of the seminiferous tubules (Hess et al, 1997a). This progressive pathogenesis is typically observed experimentally, although more rapidly, when efferent ductules of the testis are occluded after toxicant exposure or surgical ligation (Hess, 1998; Ilio and Hess, 1994). Thus, histopathogenesis that is revealed in efferent ductules of the ERKO male provides clues to understanding the infertility seen in these males.

The accumulation of fluid in the ERKO efferent ductules (Hess et al, 1997a) suggested that these ductules may


Blind ending
Rete junction
Efferent ducts
Epididymis
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WT
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be more important in male reproduction than simply acting as conduits of sperm for rapid transport from testis to epididymis. Therefore, the present study was undertaken to extend the original observations by investigating the extent of histopathogenesis in the excurrent ducts of the male reproductive system in ERKO mice. Morphologic features of noncilated cells in the efferent ductules are shown to be severely compromised, along with other morphologic parameters that are quantitatively altered in comparison to wild-type (WT) littermates. In the epididymis, the narrow and apical cells also are shown to be abnormal. Thus, pathogenesis of the reproductive tract in ERKO males is more extensive than previously reported and raises new questions regarding estrogen's function in the male reproductive system.

Materials and Methods

Male WT and ERKO mice between 70 and 135 days of age were housed 2 per cage with free access to food and water. Animals were euthanized after sodium pentobarbital anesthesia, and the reproductive tract was fixed by vascular perfusion (Hess and Moore, 1993). After fixation, efferent ductules and epididymal tissues were excised and processed for light-microscopic evaluation in either glycol methacrylate or epoxy resin. The glycol methacrylate sections were cut 2.5-μm thick and stained with periodic acid-Schiff reaction (PAS) and counterstained with hematoxylin–eosin (Hess and Moore, 1993). Tissues for epoxy sections were postfixed in 1% osmium tetroxide containing 1.5% potassium ferrocyanide (Russell and Burguet, 1977) and embedded in Quetol 812. Epoxy sections were cut 1.0-μm thick and stained with toluidine blue for light-microscopic evaluation. In 1 study, presence of blind-ending tubules in efferent ductules and epididymis was evaluated by stereomicroscopic evaluation and microdissection. Tissues were photographed with an Olympus microscope with planapochromatic lenses, a Spot-2 camera (Diagnostic Instruments, Sterling Heights, Mich), a Macintosh G3 computer (Apple, Cupertino, Calif), Photoshop 4.0 (Adobe, Mountain View, Calif), and printed on an Epson Stylus 900 printer (Torrance, Calif).

Immunohistochemical staining for carbonic anhydrase II (CA-II) was performed on tissues fixed by vascular perfusion with cold Bouin's fluid, postfixed in Bouin's fluid for another 12 hours, then washed in 70% ethanol several times. The tissues were embedded in paraffin, sectioned, and stained. Endogenous peroxidase was inactivated by 3% H_2O_2 for 15 minutes. To unmask CA-II, sections in the citrate buffer solution (pH, 6.0) were subjected to microwave heating for 5 to 10 minutes and allowed to cool for an additional 5 minutes. Slides were then washed in phosphate-buffered saline solution (PBSS) at room temperature for 5 minutes. After microwave antigen retrieval, tissues received avidin-biotin complex blocking with the DAKO system (DAKO, Carpinteria, Calif) and then nonspecific-binding blocking with 10% normal goat serum. The tissues were incubated with primary polyclonal antibody, rabbit-antihuman CA-II (Chemicon International, Temecula, Calif) at 1:1000 dilution for 12 hours at 4°C and then washed in PBSS twice for 5 minutes each. Tissues were then incubated in biotinylated antirabbit IgG (1:50) for 45 minutes at room temperature, washed, incubated in avidin-biotin complex solution from DAKO for 30 minutes at room temperature, and visualized by the chromogen. Development time was approximately 2 minutes according to the color development of the control block. After visualization, slides were dehydrated and covered with Permount.

Histologic findings were analyzed morphometrically in tissues from 3 WT and 3 ERKO males. The digital images were analyzed by NIH Image software *(http://rsb.info.nih.gov/NIH-IMAGE).* Dilation of efferent ductules was determined by measuring the widest diameter in cross-sectional areas. Epithelial height was measured from the basement membrane to the microvillus tip in nonoblique sections of ductules. Ciliated and nonciliated cell frequencies were determined by counting cells at the apical surface between cell junctions. The number of cilia was determined by counting the number of basal bodies along the surface of ciliated cells and expressing this number per 5 μm. Nuclear height was determined by linear measurements from base to apex, and the form factor was calculated as, $F = C^2/4\pi A$, where C = circumference and A = area (Stephenson, 1990). Data were first analyzed by analysis of variance, followed by Fisher's protected LSD or the Student's *t* test. Significance was determined at $P < .05$.

The beat of cilia was evaluated by 2 different methods. First, the efferent ductules of WT and ERKO males were microdissected in culture medium and digested into epithelial plaques (Chen et al, 1998). The plaques containing 10 to 20 epithelial cells were then studied by an inverted-microscopic evaluation. In the second method, microdissected efferent ductule segments were left intact without digestion and cultured for 24 hours in

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Figure 1. Gross dissection of the efferent ductules from an estrogen receptor-α knockout mouse (ERKO) male is shown attached to the initial segment epididymides, but cut away from the rete testis. Arrows point to 2 large blind-ending efferent ductules, 1 growing at a right angle to the normal direction of the ductules. The ductules were further dissected to reveal that the ductules terminate at the ends, rather than looping back (not shown). Bar = 1 mm.

Figure 2. Light micrographs of blind-ending efferent tubules (—) from wild type (WT) and ERKO males. WT blind-ending tubules **(a)** are easily identified by their darker staining appearance, smaller diameter (with the lumen nearly closed) and typical separation from other ductules by a connective tissue sheath. In the micrograph of a WT blind-ending tubule at higher magnification **(b)**, ciliated and nonciliated cells line the epithelium, but few cytoplasmic organelles typically found in normal epithelium are present. Lysosomes are absent and endocytotic vesicles are only rarely seen scattered in the cytoplasm of nonciliated cells. ERKO blind-ending tubules are also separated from normal ductules **(c)** by a connective tissue sheath. These tubules are smaller in diameter than WT, but are similar in staining intensity. In the micrograph of a ERKO blind-ending tubule at higher magnification **(d)** ciliated and nonciliated cells are present, but the cytoplasm is devoid of endocytotic vesicles and lysosomes. Arrows indicate the connective tissue sheath; C, ciliated cells; N, nonciliated cells; and L, lumen. Bar = 10 μm in a and c. Bar = 50 μm in b and d.

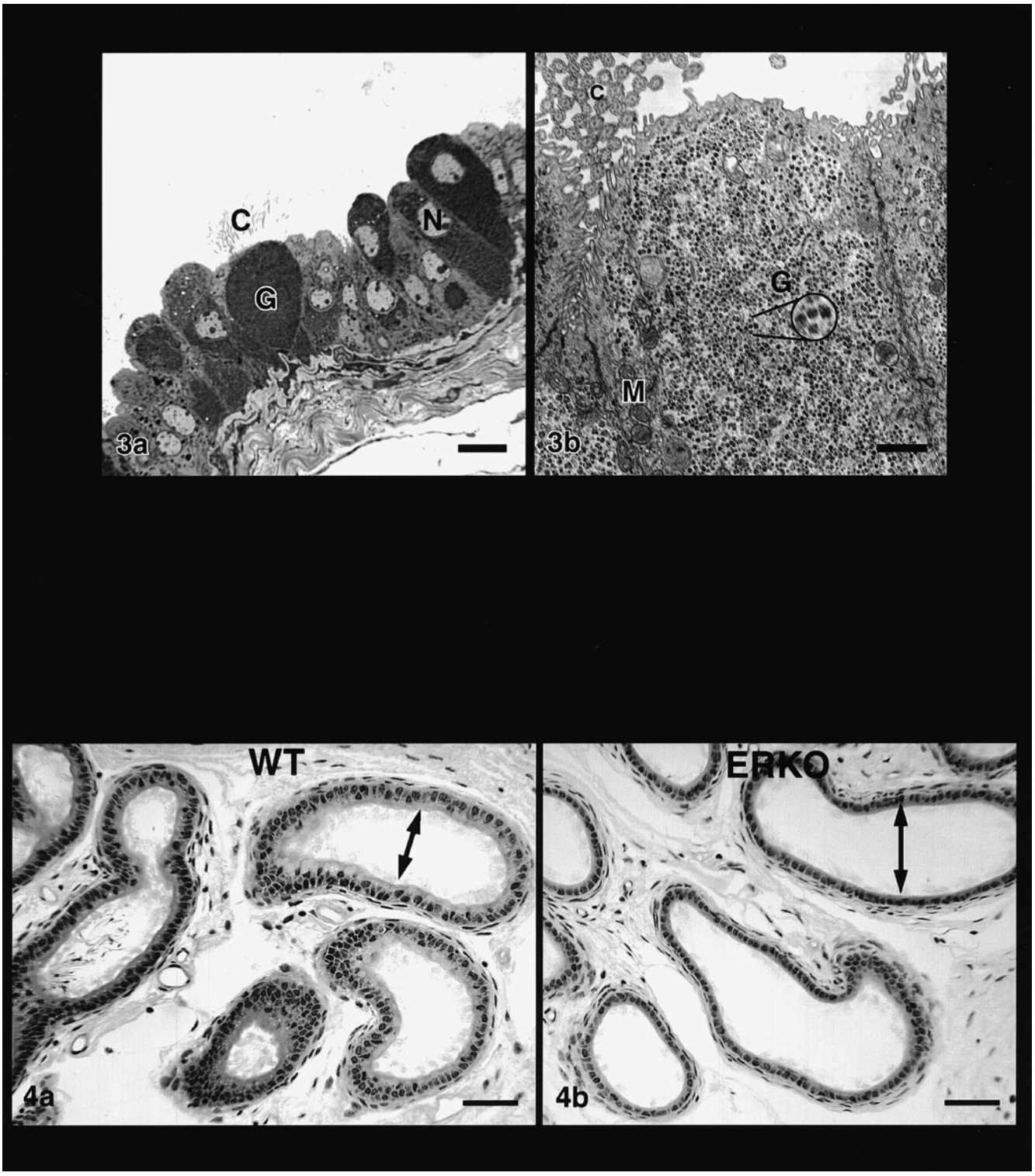

Figure 3. At the rete testis–efferent ductule junction in ERKO tissue **(a)**, cells are swollen with glycogen particles. Electron micrograph **(b)** shows a glycogen-containing cell. Mitochondria are pushed to the cell periphery by the glycogen particles. The oval inset shows an enlargement of the glycogen rosettes. G indicates glycogen; C, cilia; N, nucleus; and M, mitochondria. Bar = 10 μm in a. Bar = 1 μm in b.

Figure 4. WT efferent ductules in the proximal zone **(a)**. The epithelium is taller but the luminal diameter is smaller than ERKO ductules. ERKO efferent ductules in the proximal zone **(b)**. Bar = 50 μm.

Comparison of luminal diameters in efferent ductules of wild-type (WT) and estrogen receptor-α knockout mouse (ERKO) mice†*

Mouse type	Proximal	Conus	Common
WT	127.5 ± 11.2[a]	55.9 ± 4.7[b]	21.2 ± 1.2[c]
ERKO	293.5 ± 30.3[d]	99.7 ± 4.8[e]	84.2 ± 4.8[e]

* Means (μm) ± SEM; n = 3 animals per treatment.
† Significant differences are denoted by differences in superscripts ($P < .05$).

the medium (Chen et al, 1998). With a 40× objective, it was possible to observe ciliary beat. Beat frequency was not determined, but a metachronal wave could be observed in the WT samples.

Results

Blind-Ending Tubules

Microdissection of efferent ductules and epididymis revealed an increased incidence of blind-ending tubules in ERKO males (Figure 1) compared with WT. In ERKO tissues, 100% of the samples (10/10) in 1 study contained 1 or more blind-ending tubules compared with 40% (4/10) in WT males. In ERKO tissues, these abnormal tubules grew larger than in WT and showed greater variation in structure. In ERKO males, the blind-ending tubules often contained enlarged bulbous endings, which were never observed in the WT. Several tubules were observed growing at right angles to the efferent ductules and extended approximately 4 mm in length into the epididymal fat pad (Figure 1). These abnormal tubules were seen originating from rete testis, all regions of the efferent ductules, and the initial segment epididymidis. In WT (Figure 2a) and ERKO (Figure 2c), there was considerable similarity in the histologic attributes of the blind-ending tubules. The tubule lumen was empty and collapsed (Figure 2b and d). Epithelial cells were stained more intensely than normal and contained fewer cytoplasmic organelles. In particular, there were fewer lysosomes and endocytotic vesicles. WT mouse epithelium contained more vesicles (Figure 2b) than did ERKO (Figure 2d).

Rete Testis Junction

At the rete testis–efferent ductule junction in ERKO males, small clusters of epithelial cells were overgrown, and their cytoplasm was often saturated with glycogen (Figure 3a). The granules were PAS+ in light-microscopic evaluation and exhibited typical ultrastructural rosettes (Figure 3b). No glycogen granules were observed in any of the control tissues.

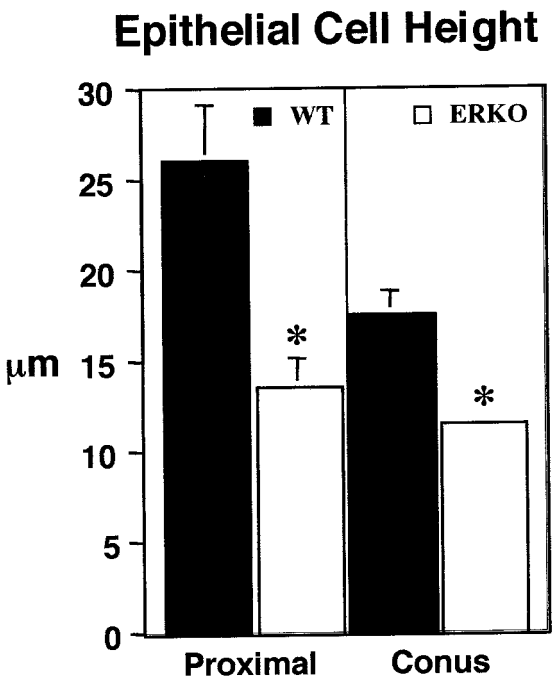

Figure 5. Epithelial cell height in the proximal and conus zones of the efferent ductules of the testis from WT and ERKO males. Significant decreases in height are noted in ERKO ductules in both regions ($P < .05$).

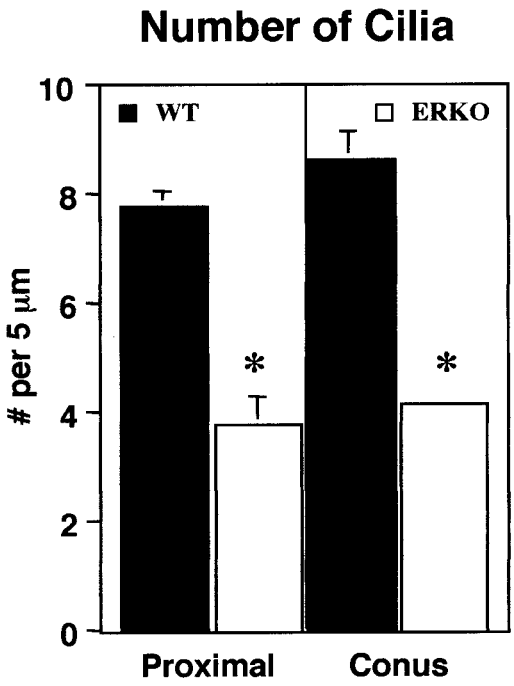

Figure 6. The number of cilia per 5 μm of apical cytoplasm in proximal and conus regions of WT and ERKO efferent ductules. Significant decreases in the number of cilia are noted in ERKO tissues ($P < .05$).

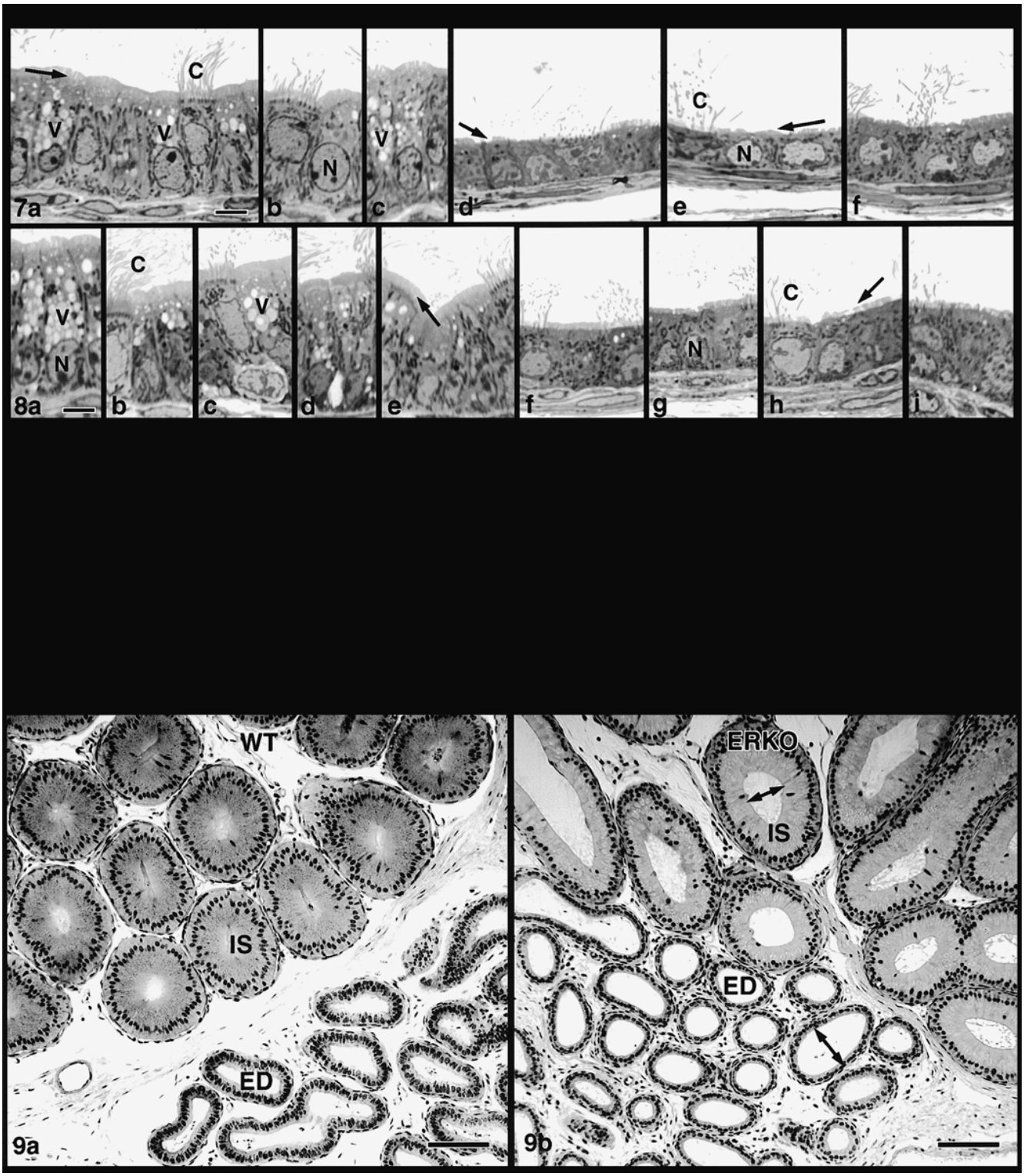

Figure 7. Proximal efferent ductule epithelium of WT **(a–c)** and ERKO **(d–f)** males, showing variation in cell types. In WT ductules, some nonciliated cells contain abundant endocytotic/lysosomal vesicles, while others appear quiescent. A thick brush border of microvilli are **(a)** in comparison to the sparse covering of short microvilli in ERKO tissues **(d,e)**. Ciliated cells in WT have more ciliary bodies that do ERKO cells and the cilia appear to bend in a metachronic wave in WT **(a)**, but appear disorganized in ERKO **(6d–f)**. V indicates vesicles; arrows, microvilli; C, ciliated cells; and N, nucleus. Bar = 5 μm.

Figure 8. Conus efferent ductule epithelium of WT **(a–e)** and ERKO **(f–i)** males, showing the differences in cell types. As in the proximal region, there is considerable variation between cells. In WT ductules, some nonciliated cells contain an abundance of large endocytotic vesicles, while others are nearly devoid of vesicles. The brush border microvilli are also quite thick and long **(e)**, compared to the short microvilli in ERKO tissues, which are

Efferent Ductule Dilation

The efferent ductules of ERKO males (Figure 4b) were dilated excessively compared with ductules observed in the WT controls (Figure 4a). Luminal diameters were increased in ERKO ductules by 130%, 78%, and 297% for the proximal, conus, and common regions, respectively (Table 1). The common region of efferent ductules (sometimes called terminus) is the single duct that is formed by the merging of multiple coiled ductules from the conus region (Ilio and Hess, 1994). The common ductule enters the initial segment epididymides. In WT animals, luminal diameters were different between all 3 regions. However, in ERKO males, there was no difference in diameters between the conus and common regions. The proximal region was threefold greater in diameter than the distal regions in the same animal

Efferent Ductule Epithelium

Epithelial cells of the efferent ductules in ERKO males were reduced in height by approximately 48% (Figure 5). The proximal region had a greater decrease in height than did the conus, but both regions were significantly shorter compared with WT cells. There was no difference between WT mice and ERKO in the percentage of ciliated cells. However, the number of cilia per cell, determined by the number of basal bodies per micrometer of apical cell surface, was reduced in ERKO tissues (Figure 6). Observations of ciliary beat in vitro revealed that cilia in ERKO tissue did not display a normal metachronal wave effect that is expected of a ciliated epithelium (Wong et al, 1993). Instead, the cilia appeared to beat randomly, each cilium being somewhat out of synchrony with the others. WT cilia beat synchronously but with a twisting motion, like the fingers of a hand rotating together, rather than waving in a straight motion.

In the WT efferent ductules, cilia extended in parallel arrays from distinct basal bodies of the apical cytoplasm (Figure 7a through b). However, in ERKO efferent ductules, cilia appeared with random cross sections (Figure 7e through f), with little evidence of a parallel arrangement that would be expected if cilia were beating in a metachronal wave. Considerable variation was noted in the nonciliated cells from WT efferent ductules (Figure 7a through c and Figure 8a through e). In WT ductules, some cells contained abundant endocytotic/lysosomal vesicles throughout the apical cytoplasm, whereas in other cells these organelles were sparse (Figure 7b). Nevertheless, when compared with WT ductules, the endocytotic apparatus in ERKO was missing or greatly reduced (Figure 7d through f and Figure 8f through i). Associated with this loss of endocytotic apparatus, microvilli along the apical border of nonciliated cells also were missing on some cells and when present were reduced in length by 64% ($P < .05$). The length of microvilli averaged 1.8 $\pm$ 0.23 μm and 0.65 $\pm$ 0.06 μm on WT and ERKO nonciliated cells, respectively. Nuclei of the epithelial cells in ERKO ductules also were found to be reduced in height, compared with WT nuclei (4.7 $\pm$ 0.5 μm vs 6.3 $\pm$ 0.2 μm, respectively; $P < .05$), but the calculated form factor for changes in nuclear shape indicated no difference between the 2 groups.

Epididymis

In ERKO tissues, luminal diameter of the initial segment region also was dilated, as was the common zone of the efferent ductules (Figure 9). Initial segment epithelium was often found displaced in the ERKO male tract. Initial segment epithelium was found growing adjacent to the proximal efferent ductule and rete testis epithelia (Figure 10a). The epididymal epithelium was approximately threefold taller than in the efferent ductule. Its identity was confirmed with electron-microscopic evaluation, which showed long, branched microvilli and a prominent Golgi apparatus, typical of principal cells (Abe et al, 1983; Abou-Haila and Fain-Maurel, 1984; Hermo et al, 1992) in the initial segment (Figure 10b). In WT males, the initial segment epithelium abruptly begins where the efferent ductule terminates under the connective tissue capsule of the caput epididymides, away from the rete testis. Displacement of initial segment epithelium also was observed in the common efferent ductule (Figure 11a and b), where spotty areas contained initial segment epithelial cells among the efferent ductal epithelium in ERKO tissues, but not in WT tissues.

Specialized epithelial cells of the epididymis, the narrow, apical, and clear cells, showed more obvious effects due to the ERα disruption in ERKO males, than was seen in the principal cells that line the different regions. However, the effects were inconsistent, some regions of the tract showing changes, but other zones showing no obvious effects when observed by light-microscopic evaluation. The effects also appeared to become more widespread with increases in age of the animal. Figure 12a shows the normal appearance of the apical and narrow

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often missing **(h)**. Cilia are more numerous in WT that in ERKO ciliated cells. V indicates vesicles; arrows, microvilli; C, ciliated cells; and N, nucleus. Bar = 5 μm.

Figure 9. In the WT male **(a)**, lumens of the initial segment and common efferent ductule are small. In the ERKO male **(b)**, the lumens are dilated. IS indicates initial segment; ED, efferent ductule. Bar = 100 μm.

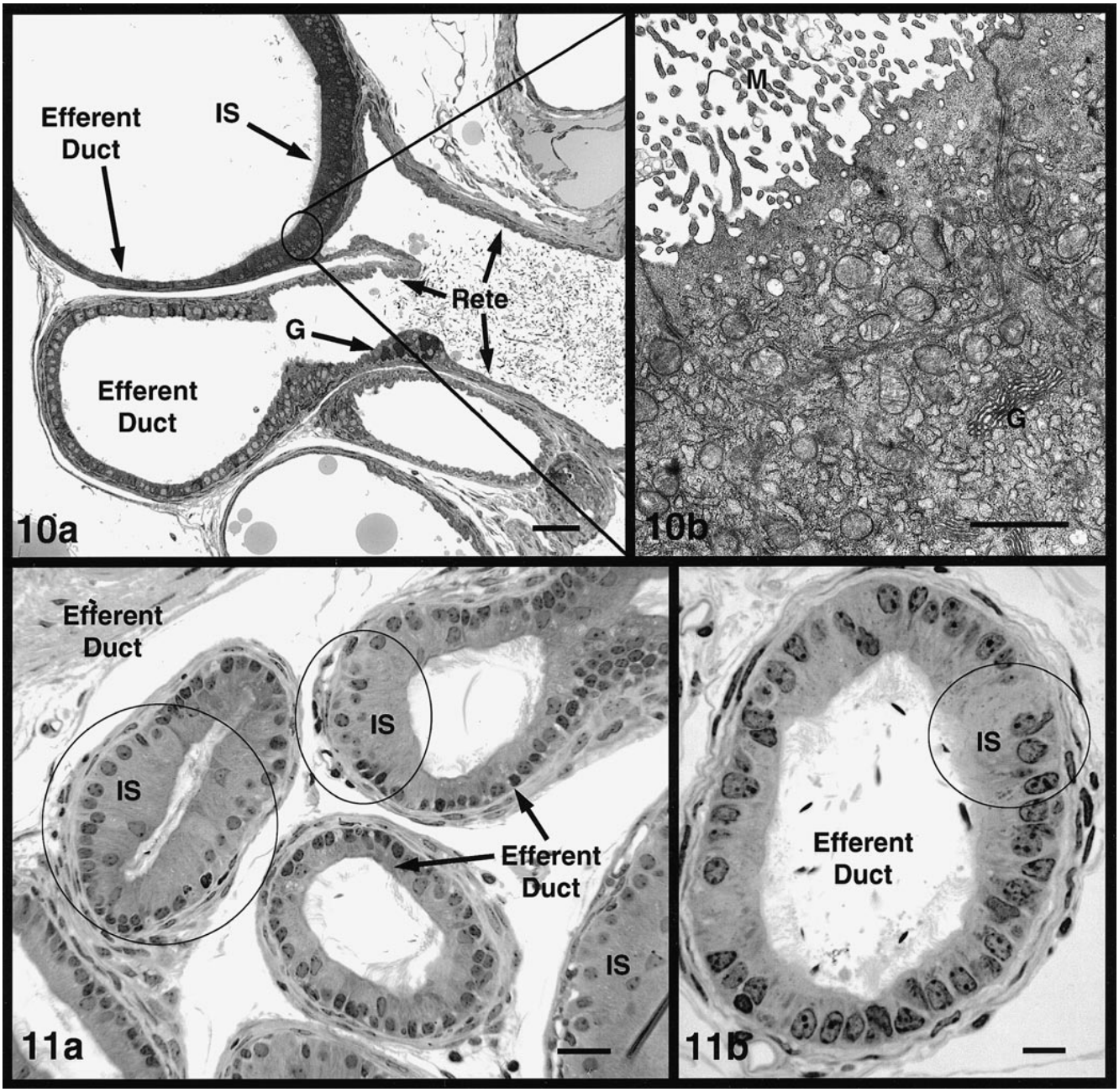

Figure 10. Low magnification of the rete testis-efferent ductule junction in ERKO male **(a)**. A group of cells containing glycogen are seen at the entrance of the efferent ductule. Initial segment epithelium is seen growing adjacent to the rete and efferent ductule. Electron micrograph of the initial segment epithelium **(b)** shown in Figure 11. Features of the principal cell are recognized by the long, thin branched microvilli and prominent Golgi apparatus. G indicates Golgi apparatus; IS, initial segment; and M, microvilli. Bar = 40 μm in a. Bar = 1 μm in b.

Figure 11. The common efferent ductule of ERKO male **(a)** contains small patches of epithelium identical to that seen in the initial segment epididymidis. Higher magnification of an initial segment patch **(b)** within a common efferent ductule epithelium of an ERKO male. IS indicates initial segment. Bar = 50 μm in a. Bar = 10 μm in b.

cells in WT epididymis. The first anomaly recognized in ERKO epididymal epithelium was a cell that accumulated excess amounts of PAS+ material in its cytoplasm (Figure 12g). At first, these cells were considered abnormal clear cells, but on further evaluation it was concluded that they were abnormal narrow cells. The nucleus was always located apically rather than basally, was sometimes triangular in shape (compare Figures 12a and b with Figure 13a), and the cell stained positive for CA II (data not shown). Furthermore, these abnormal cells only were found in the caput region, approximately segments 1 and 2 (Abou-Haila and Fain-Maurel, 1984) and not among the

more prominent population of clear cells in the cauda. The large PAS+ granules found in these cells often crowded the nucleus and protruded into the lumen. Principal cells of the epididymis also showed moderate variation. Those adjacent to the abnormal apical cells in ERKO males contained abnormally large vesicles (Figure 12g), whereas the principal cells of the initial segment surrounding the abnormal narrow cells (Figure 12c and d) were identical in structure to WT cells (Figure 12a).

In ERKO tissue from males greater than 100 days of age, 1 cell type of the initial segment epithelium protruded abnormally into the lumen and often 1 cell was seen forming a cap over 2 or 3 underlying cells (Figure 12c and d). These cells also were identified as narrow cells based on the following characteristics: 1) the cells in ERKO were positive for CA-II by immunohistochemical analysis (Figure 12e and f); and 2) narrow cells in control animals were positive for CA-II (Figure 12b), similar to that reported for the rat and mouse (Adamali and Hermo, 1996; personal communication with Dr Louis Hermo). However, with electron-microscopic evaluation, these cap cells did not contain the cup-shaped vesicles noted in narrow cell. The cells did contain numerous mitochondria and a Golgi apparatus that was located adjacent to the nucleus. This unusual appearance of the narrow cells was seen only in the initial segment region. There was no evidence that these cells were released into the lumen, although the cap cell did occasionally appear thin and pycnotic (Figure 12c). The cap cell did not contain the normal extension of long, branched microvilli that was seen in WT epithelium (Figure 12a).

The clear cells also were altered in appearance in ERKO tissue, particularly in the cauda region where they are found in abundance in WT epididymis. In WT tissues, clear cells contained numerous vacuoles in their apical cytoplasm and PAS+ granules in their basal cytoplasm (Figure 13a). In ERKO tissues, the clear cells were similar in size to those in WT, but vacuoles and PAS+ granules were nearly two- to threefold greater in diameter (Figure 13b). Principal cells of ERKO cauda epididymides were similar in appearance to WT cauda epididymides, although there tended to be more PAS+ material in the cytoplasm along the basement membrane.

The cauda epididymides showed no difference in diameter between WT (Figure 14a) and ERKO males (Figure 14b). However, the luminal contents were obviously different, as the ERKO tissue showed a greatly reduced concentration of sperm and an increase in PAS+ material between sperm (Figure 14b). In ERKO tissues, sperm were more concentrated in the distal cauda than in the proximal cauda, suggesting that the cauda epithelium may be trying to compensate for the diluted semen by reabsorbing more fluid. In 3 out of 10 males in 1 study, sperm granulomas were observed in the corpus to proximal cauda epididymides (Figure 15). It was unclear from the histologic sections how sperm became trapped in the interstitium.

Discussion

This study provides further evidence of estrogen's importance in the male reproductive tract. After disruption of ERα in the ERKO male, it was previously shown that rete testis and efferent ductules were dilated and abnormal in function (Hess et al, 1997a). In the present report, other regions of the male reproductive tract also were found to be abnormal in structure. Abnormal growth of efferent ductules and initial segment epididymal epithelium was found, suggesting that some adult dysfunctions leading to infertility (Eddy et al, 1996; Lubahn et al, 1993) may be attributed to the lack of ERα during development. Variations in epithelial cells of the ERKO epididymis were inconsistent and appeared to be exacerbated with age. Major effects were noted in the specialized cells that are scattered in specific regions along the epididymis. It is noteworthy that these specialized cells (the narrow, apical, and clear cells) have been shown to bind estradiol or express ER (Drews et al, 1988; Hess et al, 1997b; Iguchi et al, 1991; Palacios et al, 1993; Schleicher et al, 1984).

Blind-ending tubules that originate from rudimentary mesonephric tubules are found in numerous species (Blom and Christensen, 1960; Foley et al, 1995; Goyal, 1983; Guttroff et al, 1992; Hemeida and McEntee, 1984). Two kinds of aberrant ductuli were distinguished by Hemeida and McEntee (1984): 1) blind-ending tubules that originate from the rete testis and the testicular segment of the efferent ductules, and 2) blind aberrant ductules that arise from the epididymal segment. In the ERKO mouse, both types of abnormalities and growth from the efferent ductules were observed. Growth of these blind-ending tubules was more extensive in ERKO than in WT males. It is thought that aberrant tubules may cause spermiostasis in some large animal species (Ashdown and Ford, 1967), but in the rodent there is no evidence to support the development of severe pathogenesis (Guttroff et al, 1992). Nevertheless, the ERKO male may serve as a developmental model for the study of factors that induce the formation of blind-ending tubules.

The rete testis–efferent ductule junction in ERKO males contained a short segment of efferent ductule epithelium, having cells filled with glycogen granules. Such accumulation of glycogen is rarely found in highly differentiated cells of the reproductive system, although Fawcett and Dym (1974) reported similar type cells in the proximal region of the rete testis and the tubulus rectus in the guinea pig. As seen in their report, the accumulation of glycogen within the cell cytoplasm was sufficient

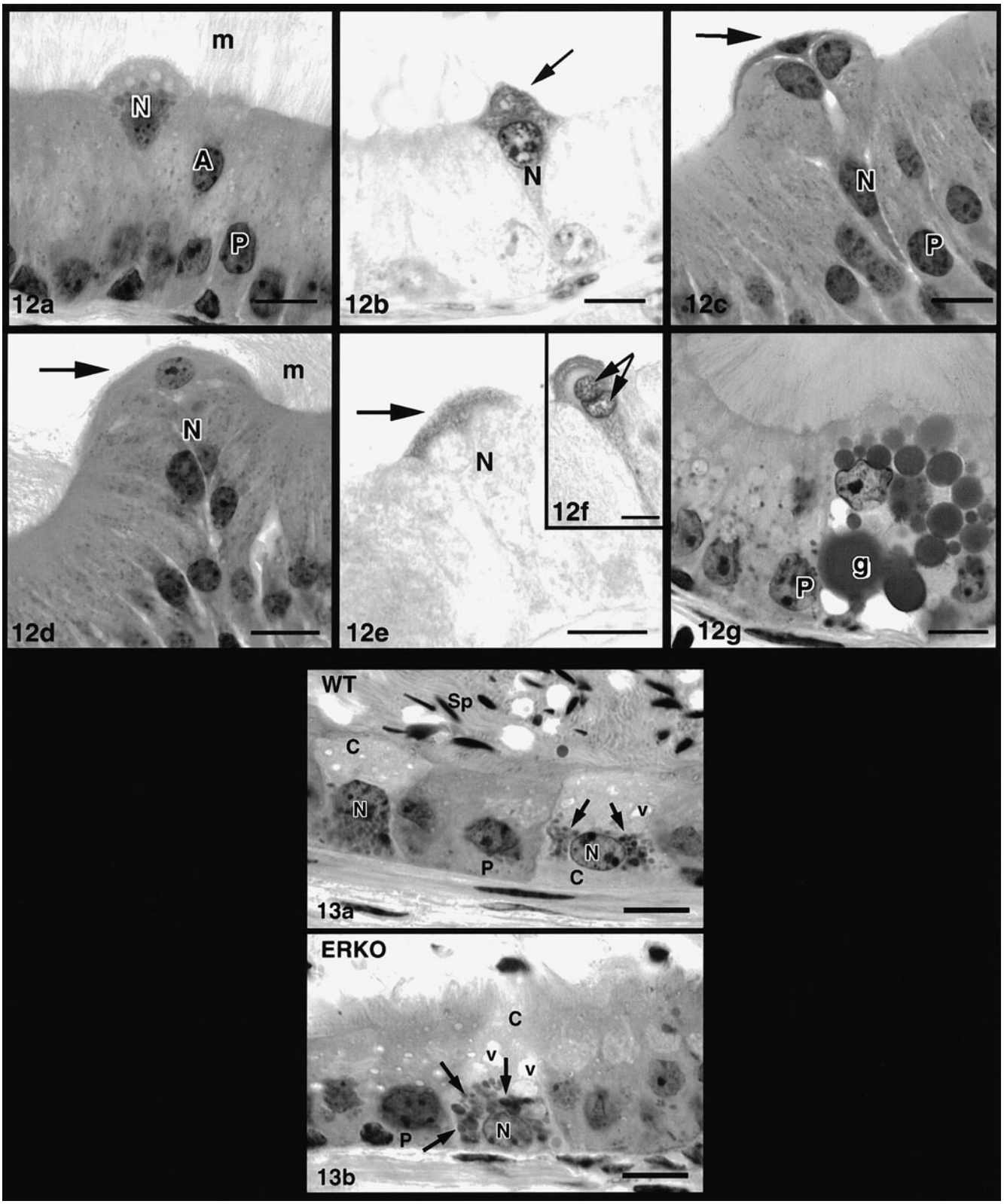

Figure 12. Initial segment epididymides in a control WT male. Long microvilli extend into the lumen from the principal cell. Apical cytoplasm of the narrow cell protrudes into the lumen and contains vesicles. The apical cell does not protrude into the lumen. Immunostaining for carbonic anhydrase II (CA-II) in the narrow cell **(b)** of the initial segment epididymidis of a WT male. Note apical cytoplasmic protrusion. Initial segment epididymidis in an ERKO male showing abnormal growth of narrow cells to form a pyramid shaped protrusion into the lumen **(c)**. A thin cell forms a tight cap over the

to displace the nucleus and to crowd other organelles into the cell periphery. Because of their rare occurrence in adult male reproductive tract, it is unlikely that these cells have a normal adult function. Instead, it is possible that these glycogen-containing cells are remnants of mesonephric tubules, left over from development. Glycogen accumulations have been noted in epithelia of mesonephric tubules and ductules (Martino and Zamboni, 1966; Pelliniemi et al, 1983; Tiedemann, 1971) and are common during development in other tissues as well (Winkler and Wille, 1998). However, glycogen is often overlooked in histologic sections, when the tissue is not preserved by proper fixation and staining (Pelliniemi et al, 1983). Glycogen's role in developing tissue is not known, but it may be important in cells that are undergoing rapid proliferation, as glycogen accumulation is observed during the preinvasive phase of testicular germ cell tumors (Kimura et al, 1998). In the ERKO male reproductive tract, fluid accumulates due to the lack of reabsorption in the efferent ductules (Hess et al, 1997a). Therefore, it is possible that stretching of the tubular system due to the build up of fluid places an increased demand on cellular proliferation, such that a small region of the mesonephric tubule fails to differentiate into a normal ductal epithelium.

ER are essential for normal structure and function of the efferent ductules of the testis (Hess et al, 1997a). In the ERKO male, efferent ductules were unable to reabsorb luminal fluids, which caused fluid to accumulate in the reproductive tract and testis. In the present study, the magnitude of efferent ductule dilation was found to range from an increased diameter of 130% in the proximal efferent ductules to nearly 300% in the common ductule. It appears that the ductules are capable of dramatic changes in structure as they respond to the loss of fluid reabsorption. However, it is interesting that it takes nearly 40 days for testicular swelling to reach maximum size (Hess et al, 1997a), which would account for the delay in achieving azoospermia in the cauda epididymidis (Eddy et al, 1996). Thus, it is possible that disruption of efferent ductule function, which leads to an overall back pressure of fluid in the ductules and seminiferous tubules, causes the long-term atrophy of the testis. Obstruction of efferent ductules experimentally initiates rapid swelling of the testis, extensive degeneration of the seminiferous epithelium, and subsequent testicular atrophy and irreversible infertility (Cooper and Jackson, 1972; Harrison, 1953; Hess et al, 1991; Kuwahara, 1976; Nakai et al, 1992).

The efferent ductal epithelium in ERKO tissue was reduced in height by nearly half, indicative of the tremendous loss in cytoplasmic organelles, a flattening of the nucleus, and the loss or shortening of the microvillus border. All of these changes are consistent with the observed decrease in fluid reabsorption by these ductules in the ERKO male (Hess et al, 1997a). The most obvious morphologic change was the loss of nearly all of the endocytotic apparatus, including apical vesicles and PAS+ lysosomal granules, which are prominent in nonciliated cells of normal efferent ductules in all species and required for normal reabsorption of luminal fluids (Hermo and Morales, 1984; Ilio and Hess, 1994; Morales and Hermo, 1983).

The microvillus border also was dramatically reduced in height in ERKO nonciliated cells, thus adding to the possible target organelles that are regulated by estrogen in the male reproductive tissues. Microvilli are fingerlike extensions of the cell plasmalemma, which contain actin microfilaments and a variety of proteins (ezrin-radixin-moesin family) that bind actin to the cell membrane (Hugo et al, 1998; Lange et al, 1997; Murthy et al, 1998). Changes in the ultrastructure of microvilli are associated with alterations in absorption (Sakai et al, 1998), therefore it is not surprising to find such changes in the ERKO male. There are now several studies that show a relationship between estrogen treatment and changes in cell ultrastructure and cytoskeletal proteins (Antakly et al, 1980; Coutts et al, 1996; Ediger et al, 1999; Vic et al, 1982). In the treatment of ER-positive breast cancer cells, Vic et al (1982) found that the number and length of microvilli could be increased by estrogen treatments. Microvillar shape also is dependent on a close relationship between actin microfilaments and Ca^{2+} (Lange et al, 1997), which makes a case for estrogen involvement because of the

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other cells. Initial segment epididymidis in an ERKO male showing abnormal growth of narrow cells **(12)**. In this case the cap cell nucleus is less pycnotic. Immunostaining for CA-II in the capping narrow cell of the initial segment epididymidis of an ERKO male **(e)**. The other narrow cells beneath the capping cell did not stain positive. Immunostaining for CA-II in the capping narrow cells of the initial segment epididymidis of an ERKO male **(f)**. The cell is binuclear and may represent an earlier form of the capping process seen in Figures 12c and d. Caput epididymidis, approximately zone II in an ERKO male **(g)**. Long microvilli extend into the lumen from the principal cells. An unidentified cell is abnormally filled with periodic acid-Schiff stained (PAS+) granules that distort the shape of the nucleus. Principal cells in this region of the tract contain numerous vesicles (arrow) that are larger than in WT tissue. Bar = 10 μm. M indicates microvilli; P, principal cell; N, narrow cell; A, apical cell; arrow, cap cell; and g, PAS+ granules. Bar = 10 μm in a through e and g. Bar ; 5 μm in f.

Figure 13. Cauda epididymidis in a WT male **(a)**. The lumen is concentrated with spermatozoa. The thin epithelium is lined by principal cells and clear cells. The clear cells contain small vesicles in the apical cytoplasm and PAS+ granules in the perinuclear region. Cauda epididymidis in an ERKO male **(b)**. The lumen contains fewer sperm than in WT. The epithelium is slightly taller, but the principal cells and clear cells are similar to those seen in WT. However, the clear cells contain larger vesicles and PAS+ granules. Sp indicates spermatozoa; P, principal cells; C, clear cells; v, vesicles; arrows, perinuclear region; and N, nucleus. Bar = 10 μm.

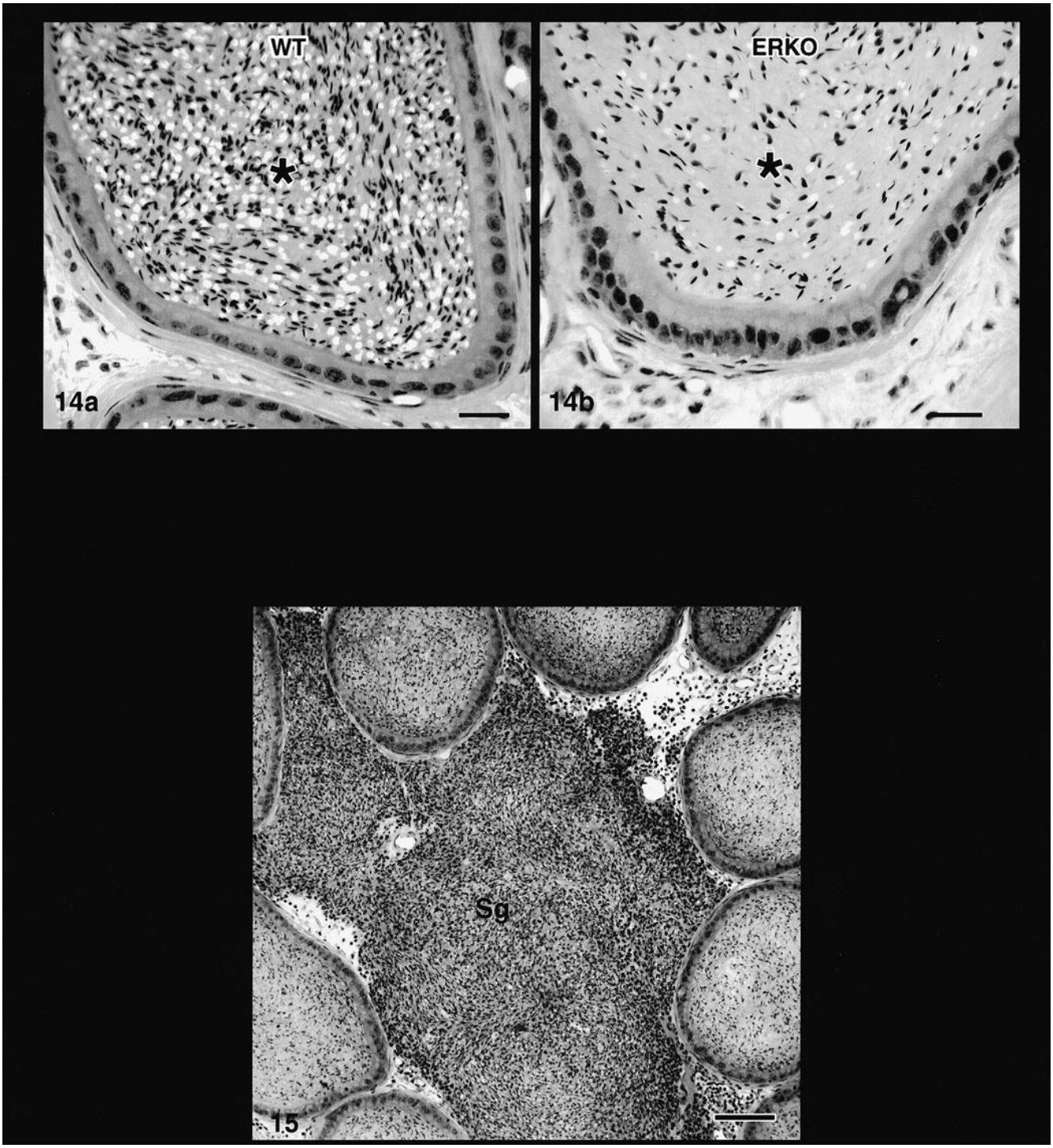

Figure 14. Low magnification of the distal cauda epididymidis in a WT male **(a)**. The lumen is filled with sperm that are concentrated by the normal fluid reabsorption along the reproductive tract. In this region there were 283 sperm heads per 100 μm^2 area of lumen. Low magnification of the distal cauda epididymidis in an ERKO male **(b)**. The lumen contains sperm that are less concentrated compared to WT. In this region there were 110 sperm heads per 100 μm^2 area of lumen. * indicates lumen. Bar = 25 μm.

Figure 15. ERKO corpus epididymidis. A sperm granuloma is seen between cross sections of the epididymal tubule. The granuloma consisted of sperm surrounded by neutrophilic leukocytes and macrophages. Sg indicates sperm granuloma. Bar = 100 μm.

well-known importance of estrogen in Ca^{2+} homeostasis (Benten et al, 1998; Dick and Prince, 1997; Liel et al, 1999; O'Loughlin and Morris, 1998). Thus, the microvillus brush border may be directly or indirectly regulated by estrogen in the efferent ductules of the male, a hypothesis that will be addressed in future experiments. This hypothesis is opposite to what is known regarding the maintenance of microvilli in the epididymis, where androgens have been shown to stimulate microvillus growth (Tezon and Blaquier, 1981).

Our first observations of ciliated cells did not suggest an effect of ER disruption. Cilia from ERKO were present and beating when isolated in culture medium (Hess et al, 1997a). However, when the number of ciliary basal bodies were counted per unit length of apical cytoplasm, it was found that ERKO ciliated cells contained half as many cilia compared with WT males. This effect on cilia is not surprising considering the fact that in the female, ciliogenesis is under estrogen regulation (Comer et al, 1998; Odor et al, 1980; Verhage et al, 1979). The results reported here for ERKO males support the previous studies that found ciliated cells of efferent ductules to be ER-positive (Ergun et al, 1997; Fisher et al, 1997; Hess et al, 1997b; Iguchi et al, 1991). The present data would suggest that estrogen also might help to regulate ciliary beat.

Abnormal growth of initial segment epithelial cells adjacent to the proximal efferent ductules and rete testis and among the common efferent ductal epithelium was unusual. Not only do these regions differentiate at different times in the rodent (Hermo et al, 1992), efferent ductules and epididymis have been considered to be of different origins. Efferent ductules are derived from the mesonephric tubules, which are thought to join the Wolffian duct (Torrey, 1943), which has been considered the epididymal derivative. However, the work of Marshall (1979) suggested that the caput epididymidis was derived from the mesonephric tubules, similar to the efferent ductules. The ERKO data would support the common origin of initial segment epithelium and efferent ductule. However, in the ER-disrupted animal, other developmental regulating factors may be abnormal, such as the Hox genes (Podlasek et al, 1999), which are involved in segmental growth and thus could determine the segmental expression of epididymal and efferent ductule epithelial genes.

The epididymis did not show a consistent pathogenesis in the ERKO male. However, abnormalities were noted in the specialized cells that are routinely ER-positive in binding studies and with immunohistochemical analysis (Drews et al, 1988; Hess et al, 1997b; Iguchi et al, 1991; Palacios et al, 1993; Schleicher et al, 1984). The apical cells and narrow cells are not the same cell type (Adamali and Hermo, 1996). Apical cells contain Yf subunit of glutathione S-transferase, but no reaction is found in the narrow cell, at least in rats (Adamali and Hermo, 1996). The narrow cells are exclusive in their expression of CA-II (Adamali and Hermo, 1996; Cohen et al, 1976). It is speculated that in the normal epididymis the narrow cells function to acidify luminal contents of the proximal epididymidis (Abou-Haila and Fain-Maurel, 1984; Au and Wong, 1980). In the present study, the narrow cells were abnormal in a specific region of the initial segment epididymidis of ERKO mice. In the mid- to distal-initial segment epididymides of ERKO, narrow cells were stacked in an unusual formation, which was capped by a thin cell. These abnormal narrow cells were identified by immunostaining for CA-II. However, they did not show the normal presence of cup-shaped apical vesicles that are normally present in the narrow cell (Adamali and Hermo, 1996). Thus, the narrow cells may have undergone degeneration or were abnormal in other respects, such as having reduced endocytosis.

In zone II of ERKO epididymis, what appeared to be apical or narrow cells were also abnormal. At first view, these cells appeared to be enlarged clear cells, due to the excessive amount of large PAS+ granules and vacuoles. However, these abnormalities were not similar to clear cell changes seen in the cauda epididymidis, and the nucleus was always located apically, rather than central or basal. Apical cells normally bulge into the lumen and show evidence of apical endocytotic vesicles and PAS+ granules, apparently associated with their suggested function in fluid uptake and protein degradation (Adamali and Hermo, 1996). In the present study, the abnormal cells were located in a specific zone of the epididymis, and they stained positive for CA-II. Thus, it is likely that the cells are an additional abnormality of the narrow cell.

Clear cells have been shown to be strongly ER-positive (Drews et al, 1988; Hess et al, 1997b; Iguchi et al, 1991; Palacios et al, 1993; Schleicher et al, 1984). However, in the ERKO male this cell type is only mildly altered in structure. They appear to have an increase in the number and size of the endocytotic vesicles and lysosomal granules. The number of clear cells did not appear to be altered. The small change in cytoplasmic organelles may be in response to the increased amount of PAS+ proteinaceous material seen in the lumen of ERKO epididymis.

The appearance of sperm granulomas in the ERKO epididymis is not understood. However, it should be pointed out that this reproductive tract lesion was one of the first to be discovered in the experimental male response to in utero diethylstilbestrol exposure (McLachlan et al, 1975).

In conclusion, the entire reproductive tract of the ERKO male, including rete testis, efferent ductules, and epididymis, is affected in this ER− animal model. The data in some cases are consistent with ER studies showing that specific cells of the epididymis are ER+. An understanding of other data, such as the displaced growth of epididymal epithelium near rete testis, awaits the discov-

ery of new estrogen regulated genes, possibly those associated with segmental differentiation. These data suggest that there are developmental anomalies in the ERKO male, as well as adult dysfunctional changes. However, we still do not know whether sperm that exit the ERKO testis are normal but lose their fertilizing capability in the efferent ductules or epididymis. An understanding of these separate effects will only come from future studies in which rodents are treated with a potent antiestrogen to block ER function in a normal adult male.

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