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Small tubules, surprising discoveries: from efferent ductules in the turkey to the discovery that estrogen receptor alpha is essential for fertility in the male

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Abstract

Efferent ductules are small, delicate tubules that connect rete testis with the head of the epididymis, first identified by de Graaf in 1668. Although difficult to find in routine dissection, the ductules are an essential component of the male reproductive tract and in larger mammals occupy up more than 50% of the caput epididymidis. My introduction to research began with the study of efferent ductules in the domestic turkey, and to my surprise these small structures with kidney-like function become the core for numerous discoveries throughout my scientific career. In this review, only two discoveries that I found interesting will be discussed: cilia that line the efferent ductule lumen and estrogen receptors that play an essential role in regulating fluid reabsorption. A potential link between these two discoveries was uncovered in the study of efferent ductule effects observed in the estrogen receptor knockout mouse and following toxic exposure to the fungicide benomyl.

Keywords

cilia; ductuli efferentes; efferent ductules; estrogen receptor; fluid resorption; ion transport; sperm granuloma

Introduction

Efferent ductules (ductuli efferentes) are small, highly convoluted and delicate tubules that connect rete testis cavities with the head of the epididymis (Fig. 1, 2, 3). In rodents the ductules are buried loosely in the epididymal fat pad (Fig. 1), but in larger mammals they occupy lobules (Fig. 3) and take up more than 50% of the caput epididymides (Yeung *et al.*, 1991; Hess, 2002). It is surprising that these small structures, with kidney-like function, could become the core scientific basis for one scientist's career and eventually be the source of inspiration for the discovery that estrogen receptor-α (ESR1) has an important role in regulating fluid reabsorption in the male reproductive tract. In male reproductive biology, the epididymis is routinely the primary focus for those interested in the transport and storage of sperm in the reproductive tract. However, an occasional study over the past 150 years has

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revealed several interesting features associated with the efferent ductules, including the fact that they are indispensible for male fertility (Table 1). More surprising to me is how often scientific inquiry drew me into the study of this ciliated epithelium. In this brief review, I would like to highlight two surprising discoveries associated with these ductules, 1) motile cilia are found lining ciliated cells of the ductal epithelium and 2) estrogen receptor regulates its major kidney-like function. A link between efferent ductule dysfunction after exposure to a fungicide and the discovery that estrogen plays a major role in regulating the kidney-like function will be presented.

Motile cilia

One of the more noteworthy features observed in efferent ductules was reported in 1856, in what appears to be the third publication to describe this region of the male tract. In this German manuscript, Becker (1856) reported that the efferent ductule epithelium contains motile cilia and is histologically distinct from the epididymis. Becker was the first to suggest that the cilia propel sperm toward the epididymal lumen and because the ciliated cell has received limited attention that original hypothesis is still promoted in many textbooks. In general there is a greater proportion of nonciliated cells in the proximal regions but an increase in the number of ciliated cells in the region nearest the epididymis. There is considerable variation in the proportion of ciliated cells (Fig. 2), ranging from 1:2 to 1:5, depending on the species and ductal region (Ilio and Hess, 1994). In a few species, ciliated cells can occupy up to 80% of the ductal epithelium (Hemeida *et al.*, 1978). Cilia extend from basal bodies as parallel fingers into the lumen (Fig. 2) and bend together in the lower half but appear to be more random near their tips (Fig. 4). A central axonemal complex grows from the basal body and rootlets are occasionally found extending from the basal body into the apical cytoplasm (Fig. 2). Numerous mitochondria are found beneath the basal bodies to support a high demand for energy when cilia are beating.

Cilium is the Latin word for eyelash and morphologically is the long finger-like protrusion with a central axonemal complex that extends from the cell body. There are two specific types: motile and non-motile (called primary cilia). Motile cilia are found in only four adult organs (Table 2): the respiratory system, ependymal epithelium of the nervous system, the female oviduct, and efferent ductules of the testis (Fig. 2). The sperm tail or flagellum is also considered a motile cilium. In the embryo, the pit of Hensen's Node at the caudal end of the notochord also supports epithelial cells with motile cilia, which is necessary for development of the body left/right symmetry. Also during development of the ear, the otic vesicle hair cell has kinocilia that vibrate and tether the otolith stones. There are over 600 proteins uniquely arranged in this structure to provide its specialized functions that are more complicated than just their ability to beat (Table 2).

Ciliary beat has been studied predominantly in the respiratory system, whose epithelium requires the ciliary beat to be coordinated in a metachronal wave formation to move mucus in a cephalic direction. I was fortunate to work in pulmonary medicine for a number of years prior to completing my graduate studies. In fact, the first publication that I co-authored in 1975 was an *in vitro* study of ciliary inhibition (Adshead *et al.*, 1975). Ultrastructure of the pulmonary cilium was fascinating to me, as it illustrated the beauty of such precise

organization of structural proteins to produce a defined function. While working in pulmonary medicine I began graduate studies in male reproductive biology of the avian domestic turkey, which was my second exposure to a ciliated epithelium, the efferent ductules (Hess *et al.*, 1976; Hess and Thurston, 1977). Ciliated cells of the turkey efferent ductule were identical to those in trachea and we assumed they had the same function, which was promoted by Becker (1856).

Inhibition of ciliary beat in trachea and bronchus contributes to infections and inflammatory responses of the respiratory system in several human diseases, which also include male infertility (Satir and Christensen, 2007; Mishra *et al.*, 2012; Busquets *et al.*, 2013). Therefore, when we first observed efferent ductule occlusions and inflammation following exposure of male rats to the fungicides benomyl and its metabolite carbendazim (Hess *et al.*, 1991; Nakai *et al.*, 1992; Hess and Nakai, 2000), one of the first hypotheses was the loss of ciliary function, as the axonemal complex is microtubule dependent and fungicides are microtubule poisons. However, the developed cilia appeared normal and the occlusions appeared to be related to the kidney-like function of the epithelium. However, it is now apparent in hindsight that even fluid resorption is also dependent on microtubule polymerization for the movement of membrane proteins and the recycling of vesicles (Elkjaer *et al.*, 1995; Morris *et al.*, 1998; Breton *et al.*, 2000; Mirela *et al.*, 2000; Rogers and Gelfand, 2000; Romano *et al.*, 2000; Chalumeau *et al.*, 2001; Pastor-Soler *et al.*, 2001; Sabolic *et al.*, 2002).

Ciliary beat in the efferent ductules was a persistent curiosity. Finally in 1987 my laboratory video recorded the ciliary beat and to our surprise observed a rotational twist, rather than a distinctly coordinated wave as observed with epithelia in trachea and the oviduct. It has been difficult finding ultrastructural evidence for rotation of the cilia, which may be explained by the small size of the ciliary tips that are approximately 100–150 nm in diameter (Fig. 5). In light and electron microscopy, the ciliary tips are located randomly and never aligned. Only an occasional section in electron microscopy reveals the twisting of microtubules, which can be seen in both longitudinal sections and cross sections near the tip (Fig. 5). Although evidence for twisting has been observed in efferent ductal cilia, how the microtubules attach and/or disengage from the capping structure and crown remains to be discovered. In respiratory and oviductal epithelia, cilia beat in a synchronized wave and when viewed with electron microscopy there is no evidence of twisting (Dalen, 1983; Portman *et al.*, 1987). Based on the observed twisting of microtubules in the ciliary tips and the video evidence of rotation, we hypothesized that this mode of action served the purpose of stirring or mixing the luminal contents, rather than pushing sperm toward the epididymis (Ilio and Hess, 1994; Hess, 2002). Such a function may be necessary for consistent resorption of fluid at the lumen/nonciliated cell interface, where up to 90% of the fluid is moved across the epithelium and metabolized, recycled or reabsorbed into the epididymal vasculature in a rapid period of time.

A rotational beat of cilia has also been found in epithelial cells lining the pit of Hensen's Node, at the caudal end of the embryonic notochord, which is essential for the development of left-right symmetry (Tabin, 2006). It has been speculated that twirling or rotational ciliary beat is found only in cilia lacking the central pair of microtubules and having an axoneme of

9+0 (Brokaw, 2005; Hilfinger and Julicher, 2008; Yoshiba *et al.*, 2012), but this has been controversial and in light of the 9+2 microtubule structure of cilia in efferent ductules, it is possible that another mechanism for the rotation is yet to be revealed. Immotility of nodal cilia results in situs inversus and if other ciliary structures are also immotile, the Kartagener's Syndrome develops, resulting in chronic pulmonary infections and infertility (Mishra *et al.*, 2012; Busquets *et al.*, 2013; Knowles *et al.*, 2013). Although sperm immobility in this syndrome is the primary cause of infertility, occlusion of the caput epididymis has been reported (Schanker *et al.*, 1985; Bashi *et al.*, 1988), which raises the possibility that immotility of efferent ductule cilia may disrupt fluid resorption and contribute to the formation of sperm granulomas.

In 1995 histopathological changes in the *Esr1* knockout mouse (*Esr1*KO) testis were first presented (Eddy *et al.*, 1996). My focus on efferent ductule structure and function (Ilio and Hess, 1994; Hess, 2002) allowed me to predict that estrogen's primary target was the nonciliated cells of the epithelium (see comments under Estrogen target). However, at the time there was considerable controversy over estrogen's regulation of ciliogenesis in the oviduct (Odor *et al.*, 1980; Reeder and Shirley, 1999; Brody *et al.*, 2000; Okada *et al.*, 2004); therefore, we investigated the efferent ductule ciliated, as well as nonciliated cells (Hess *et al.*, 2000). The number of cilia per cell was reduced by more than 50% in the *Esr1*KO (Fig. 6) and even the ciliary beat appeared abnormally random and out of synchrony. Wild-type cilia extended in parallel arrays from distinct basal bodies, while *Esr1*KO cilia appeared disorganized. There has been no follow up of these original findings, but they do raise questions regarding ciliary function in the efferent ductules and their regulation by estrogen through ESR1.

More recent discoveries have shown the importance of long conserved genes such as *Foxj1* and *Rfx3* in the control of ciliogenesis (Okada *et al.*, 2004; Choksi *et al.*, 2014), but their link to *Esr1* and estrogen's role in the ciliated epithelium remains unknown. However, their link to efferent ductule development and function cannot be ignored. Notch signaling has been linked with *Foxj1* and ciliogenesis by providing an inhibitory influence (Seeger-Nukpezah and Golemis, 2012; Stubbs *et al.*, 2012; Li *et al.*, 2013; Choksi *et al.*, 2014); therefore, it is reasonable to hypothesize that the ciliated cells could provide a regulatory cross-talk with the nonciliated cells, particularly during development. This is supported by data from the constitutive expression of Notch1 mouse, in which hyperplasia of the efferent ductules was reported, although the investigators did not report on the ciliated cells (Lupien *et al.*, 2006).

Another unique feature of efferent ductule cilia is the ciliary crown that consists of a tiny claw-like grasping complex of bristles that extend from the plasmalemma at the ciliary tip (Fig. 7). These are very rare to observe due to their size (a single claw is approximately 5 nm in width), the plane of section and quality of preservation (Portman *et al.*, 1987). Currently, it is not known if all ciliary tips have the crown and claw structures in efferent ductules and another difficulty is the fact that the tip of the sperm flagellum does not have the claw, but can appear similar to the motile cilium (Fisch and Dupuis-Williams, 2011). The ciliary crown was first reported in oviduct cilia but has now been reported also in respiratory epithelium and a few other tissues (Dirksen and Satir, 1972; Kuhn and Engleman, 1978;

Dalen, 1983; Portman *et al.*, 1987; Fisch and Dupuis-Williams, 2011). In the oviduct, the ciliary crown is required for transport of and grasping the ovum and tracheal cilia use the crown for grasping mucus (Dalen, 1983; Fisch and Dupuis-Williams, 2011). In efferent ductal epithelium, the ciliary crown's function is pure speculation, but could serve some sort of signaling function by grasping the membranes of luminal sperm.

The coordination of cell signaling pathways is a more recent function of the cilium, particularly that of the primary cilium (Satir *et al.*, 2010; Mukhopadhyay and Rohatgi, 2014). The primary cilium is a solitary finger-like extension from the cell surface of most mammalian cell types, first reported in 1968. Although once thought to be vestigial or relegated to developmental activity, the primary cilium is now recognized for its functional significance in a diverse array of cellular events, ranging from centriole sequestration, inhibition of cell division, cell polarity, mechanosensitivity, chemosensitivity and receptor-mediate cell signaling (Shah *et al.*, 2009; Bloodgood, 2010; Satir *et al.*, 2010; Takeda and Narita, 2012; Knowles *et al.*, 2013). It is interesting that for many years my laboratory observed a solitary cilium projecting from nonciliated cells of the efferent ductules (Fig. 8). The structure is always located near the tight junctional complex of the nonciliated cell and appears to be shorter in length than the motile cilium. In other tissues, the primary cilium has been reported as short stump-like structures (Satir *et al.*, 2010) and has the 9+0 microtubule arrangement (Ishikawa and Marshall, 2011), but cross sections of this cilium have not been described for efferent ductules. If the cell re-enters the cell cycle for division, the primary cilium is resorbed and then regrown from the centriole as the cell differentiates. Orientation of the primary cilium determines cell polarity and potentially self-renewal of stem cells (Veland *et al.*, 2009; Satir, 2010; Schatten and Sun, 2010). Essentially nothing is known about this organelle in male reproduction. However, one might predict an important signaling activity related to fluid flow and luminal contents, because bending of the primary cilium in the direction of fluid flow in the kidney proximal tubule is associated with calcium signaling (Praetorius and Spring, 2003) and changes in length of the primary cilium is associated with occlusions and repair of the renal tubules (Wang *et al.*, 2008).

Estrogen target

The most important feature of the efferent ductule epithelium is its basic physiological function to reabsorb up to about 90% of the luminal fluids coming from the seminiferous tubules, including 50–90% of the luminal proteins (Clulow *et al.*, 1998; Hess, 2002; Hansen *et al.*, 2004). The ductule expresses several major proteins common to the proximal tubules of the kidney, including SLC9A3, CAR2, SLC9A1, SLC26A3, CFTR, AQP1, and AQP9 (Hess *et al.*, 1997a; Lee *et al.*, 2001; Zhou *et al.*, 2001; Oliveira *et al.*, 2005; Ruz *et al.*, 2006). Its kidney-like activity involves both an energy-dependent solute exchange system, passive water permeability and a complex endocytic apparatus (Morales and Hermo, 1983; Hermo and Morales, 1984; Hermo *et al.*, 1988; Clulow *et al.*, 1998; Hess, 2002; Hermo and Smith, 2011) and occurs rapidly, as sperm traverse the efferent ducts in 45 min in the rat (English and Dym, 1982). Thus, the regulation of efferent ductule function is vitally important and has numerous pathways that could be the target of toxicity and gene disruption.

In 1973, I began my Master's degree program and was asked to study histology of the male reproductive tract in the domestic turkey, because 15% of the males produced discolored semen and were labeled with the 'Yellow Semen Syndrome'. Quickly I discovered that textbooks were not helpful and professors of histology only knew general structures and functions in mammalian species. Although I was training as a physiologist, my passion soon became histology and I enjoyed spending hours looking through the microscope. The art, the beauty and the symmetry of cells and tissues opened a new world to me, which has remained with me for more than 40 years. I finally published the first description of ciliated and nonciliated cells in efferent ductules of the turkey.

During my doctoral research, I demonstrated an abnormal accumulation of cholesterol in the yellow semen syndrome, which originated from the efferent ductule epithelium (Hess *et al.*, 1976, 1982, 1984; Hess and Thurston, 1977; Thurston *et al.*, 1982). That study was my introduction to the importance of efferent ductules in the production of fertile sperm, which eventually led to the first review article to be focused only on these small tubules (Ilio and Hess, 1994) During my postdoctoral training in reproductive toxicology at the US Environmental Protection Agency, in North Carolina, my job was the simple task of helping the laboratory to understand spermatogenesis and testicular pathology after toxicant exposures. I took these studies with me upon moving to the University of Illinois to accept a professorship in 1986. At first, my focus was to understand stages in the cycle of the seminiferous epithelium (Leblond and Clermont, 1952). At that time, a strong bias was voiced that male infertility was caused by toxicants targeting the Sertoli and Leydig cells and/or germ cells of the testis. A common fungicide, Benomyl and its metabolite carbendazim were the first chemicals I studied. In a 90-day treatment protocol, these compounds caused male infertility and testicular atrophy. Because benomyl is a microtubule poison, binding to β-tubulin, we tested the hypothesis that seminiferous tubular atrophy was simply the inhibition of mitosis in spermatogonial germ cells.

However, to understand testicular atrophy it was necessary to trace the pathogenesis back in time, which to our surprise showed massive sloughing of germ cells two days after a single exposure and testicular enlargement (Carter *et al.*, 1987; Hess *et al.*, 1991). Testis weight increased dramatically within hours post treatment and then began to atrophy (Fig. 9). The older literature on efferent ductules had already shown that occlusion of the lumen would prevent the passage of sperm from rete testis to the epididymis, causing a rapid build-up of fluid in the testis, dilation of the seminiferous tubular lumen and ultimately lead to tubular degeneration and total testicular atrophy (Hess *et al.*, 1991; Nakai *et al.*, 1992; Nakai and Hess, 1994; Hess and Nakai, 2000). It appeared that the fungicide altered fluid resorption, causing sperm stasis and a massive inflammatory response (Fig. 10). The resulting sperm granulomas eventually became permanent fibrotic lesions, although the ductal epithelium did attempt to regrow around these lesions (Nakai *et al.*, 1993). Most importantly, it was concluded that testicular atrophy following chronic or subchronic exposures to a toxicant should be re-examined for histopathological lesions in the efferent ductules and head of the epididymis and that such lesions may result in complete or partial blockage, decreases in sperm output and permanent injury to the male reproductive system.

While studying the effects of benomyl on the testis and efferent ductules, I began to speculate on hormonal regulation of the ductules. At the time, everything was focused on testosterone (T) or dihydrotestosterone (DHT; Turner *et al.*, 1985). However, one study using ^{3}H-estradiol (E2) and autoradiography showed efferent ductules having the most intense labeling in the male (Schleicher *et al.*, 1984). I remembered this when Paul Cooke, a young scientist we had just hired, asked which organ I would be interested in examining for estrogen binding in the male during development. He and Cunha were using tissue recombination methods and autoradiography to study androgen regulation of development and decided to also look for estrogen binding (Cooke *et al.*, 1991a). My contribution was minor but it changed my career. I said they should look at the efferent ductules. To our surprise, efferent ductule epithelium was the first region of the male tract to bind ^{3}H-E2 on day 16 of gestation and subsequently, with the initial segment epididymis appearing next but less than the efferent ducts (Cooke *et al.*, 1991b). That study solidified my original hypothesis that estrogen could be a major regulator of these small tubules, but there was one problem. What could be the source of estrogen?

My first thought was that the epithelium itself produced estrogen from the high concentration of T that was always found in rete testis fluid as well as semen (Free and Jaffe, 1979; Hess, 2004). It would have been simple for the efferent duct epithelium to express the aromatase gene; however, data were lacking. Soon after we published the study showing strong^{3}H-E2 binding in the efferent ductules, Hiro Nitta, a graduate student of another colleague Janice Bahr, brought several antibodies to my lab and asked if we could use them for localization of steroid-synthesizing enzymes in the male reproductive system. Most of the antibodies gave uninteresting results, but one gave us a surprise. P450 aromatase showed strong expression in germ cells of the testis (Nitta *et al.*, 1993), a discovery that contradicted thirty years of research asserting that Sertoli cells synthesized estrogen in the prepubertal male while in the adult testis only Leydig cells produced estrogen (Carreau and Hess, 2010; Hess *et al.*, 2011; Joseph *et al.*, 2011). This research allowed me to change my earlier hypothesis and to suggest that spermatozoa were mobile endocrine units, producing local concentrations of E2 that would target the efferent ductules, whose major function was rapid reabsorption of fluid to increase the concentration of sperm as they entered the epididymis. How could T regulate the resorption of fluid when it's concentration in blood and rete testis fluid was always elevated? E2 was the perfect alternative and could be produced proportional to the number of sperm coming down the tract. It was fun to speculate, but we had no evidence that estrogen receptors were actually present and functional in the efferent ductules, although they did bind E2. However, that was about to change, as we were able to show for the first time, using an antibody directed against the rat N-terminal region, that ESR1 was abundantly expressed in nuclei of the efferent ductule epithelium and its mRNA was 3.5 times greater than in the uterus (Hess *et al.*, 1997b).

At an annual scientific meeting, Mitch Eddy introduced the male estrogen receptor-α knockout mouse (*Esr1*KO), which showed massive dilation of the rete testis and seminiferous tubules (Lubahn *et al.*, 1993; Eddy *et al.*, 1996). I knew immediately that the target of estrogen was the efferent ductules and called Dennis Lubahn to collaborate on this new mouse model. The first experiment was to weigh the testis over time and data revealed a graph identical to that of benomyl (Hess *et al.*, 1997a). Although the timing of increased

testis weight was delayed, compared to benomyl, the same pattern of increase followed by atrophy was seen (Fig. 9). The earlier work with benomyl led us to speculate that the lack of *Esr1* expression in the male would cause a blockage of the efferent ductal lumen, resulting in backpressure and fluid accumulation in the rete testis and seminiferous tubules. However, histopathology revealed an opposite result. Efferent ductules of the *Esr1*KO mouse were filled with fluid, dilated excessively (Fig. 11) and fluid was accumulating in the rete testis and seminiferous tubules (Hess *et al.*, 2000; Nakai *et al.*, 2001). One of our first experiments involved the isolation of individual efferent ductules in culture, with the goal of ligating each end with suture (Hess *et al.*, 1997a). To our surprise, most sutures were larger in diameter than the efferent ductules. Finally, we unraveled the smallest suture and using one strand of fiber and making a loop, we were able to gently push the tubule end through the loop and ligate. After 12 h in culture, the wild-type ductule lumen was collapsed because water followed the Na^+ and fluid was moved into the culture medium. However, the *Esr1*KO ductules showed an increase in diameter, suggesting that not only had resorption been inhibited, but also water had flowed into the lumen, possibly due to the stimulation of the Cl^- channel (Hess *et al.*, 1997a). Subsequent studies reported an increase in the expression of the *Cftr gene* (cystic fibrosis transmembrane conductance regulator; Lee *et al.*, 2001, 2008; Toda *et al.*, 2008). Epithelial cells of the efferent ductules in the *Esr1*KO mouse and antiestrogen ICI 182,780 treated males had a dramatic loss of cellular organelles responsible for their kidney-like function (Fig. 11).

Thus, maintenance of fluid resorption by nonciliated cells of the efferent ductule epithelium (Fig. 11) is a major function of ESR1, as well as control of ciliary growth and beat. Along with several other labs, we have now uncovered numerous genes controlled by *Esr1* in the efferent ductules and much of this work confirmed its regulation of proteins responsible for ion transport and water movement across the epithelium (Hess, 2003; Carreau and Hess, 2010; Hess *et al.*, 2011; Joseph *et al.*, 2011). However, there remains one surprising observation. While performing the 24-h culture of efferent ductule segments, we accidently left a few in cultures for more than 24 h. Most of these were thrown away, but out of curiosity we examined a few under the microscope. To our surprise, some of the wild-type ductules had returned to their original diameters. We were never able to follow up this finding but it provides the basis for a new hypothesis: when motile cilia and possibly primary cilia of the nonciliated cells touch or become congested in the lumen, a signaling pathway is activated to inhibit or reverse fluid resorption by the nonciliated cell, thereby preventing occlusion of the lumen if possible.

Other laboratories are now taking up the effort to study estrogen in the male (Pearl *et al.*, 2011; Gonzalez *et al.*, 2012; Berger *et al.*, 2013; Fietz *et al.*, 2013; Arkoun *et al.*, 2014; Chimento *et al.*, 2014; Lin *et al.*, 2014; Lucas *et al.*, 2014; Toda *et al.*, 2014), which is leading to more surprising discoveries but raising many new questions for future study. One of the more perplexing questions has been why the efferent ductule epithelium is essentially normal in the aromatase knockout mouse, with the total loss of E2 synthesis (Robertson *et al.*, 2002; Toda *et al.*, 2008). Table 3 lists several possible explanations, but the one that is most intriguing is related to the potential functions of estrogen through multiple pathways, including nuclear receptor-mediated transcriptional activity, ligand dependent and independent pathways, as well as rapid effects through a membrane receptor (Revankar *et*

al., 2005; Carreau *et al.*, 2006; Filardo *et al.*, 2007; McDevitt *et al.*, 2007, 2008; Lucas *et al.*, 2008, 2010; Sinkevicius *et al.*, 2008, 2009; Weiss *et al.*, 2008; Lazari *et al.*, 2009; Carreau and Hess, 2010; Hess *et al.*, 2011). Non-classical estrogen receptors finally been accepted in mainstream endocrinology, but how these pathways interface with physiology of the classical nuclear receptors in male reproduction (Lucas *et al.*, 2011) remains an open question. Efferent ductules are unique in that they express not only androgen and estrogen receptors, but also several other nuclear steroid receptors (Hess *et al.*, 2011) and the question is raised how multiple steroid receptors compete for common cofactors and activate genes that have response elements for multiple steroid receptors? How do the efferent ductules integrate these numerous signaling pathways and how do cilia and microtubules participate in this activity?

Conclusions

I would like to conclude by drawing a speculative link between estrogen action in the efferent ductules and microtubule dysfunction seen with the fungicide benomyl. Benomyl induces fluid accumulation in the testis (Hess and Nakai, 2000), the same as seen with ESR1 disruption (Hess *et al.*, 1997a). However, benomyl causes occlusions of the efferent ductule lumen, while ESR1 dysfunction results in dilution of the lumen and swelling. There are two possible links to explain these effects: 1) Microtubules play an important role in vesicle transport, membrane recycling and turnover of membrane proteins (Elkjaer *et al.*, 1995; Breton *et al.*, 2000; Pastor-Soler *et al.*, 2001; Sabolic *et al.*, 2002; Maldonado-Baez *et al.*, 2013). Therefore, benomyl's block of microtubule polymerization would likely disrupt microtubule-dependent pathways responsible for membrane recycling of ion and water transport proteins that are normally regulated by ESR1. This would cause an increased rate of fluid resorption, sperm stasis and luminal compaction. Carbendazim has been shown to increase the activity of Na+/K+-ATPase along the basolateral border of the nonciliated cells (Ilio and Hess, 1992); 2) Microtubule polymerization is inhibited by estradiol but testosterone inhibits microtubule depolymerization (Kipp and Ramirez, 2003). That study was published in 2003 but appears to have been lost in the literature. However, that discovery directly links the effects of benomyl with estrogen's function in efferent ductules, a tissue expressing ESR1 greater than even the uterus. A microtubule inhibitor would displace the T-binding site (Kipp and Ramirez, 2003) and the endogenous high concentration of E2 in efferent ductules (Free and Jaffe, 1979) would insure the loss of nonciliated cell microtubules and result in build up of ion and water transport proteins in the membrane. Thus, efferent ductule epithelium appears to be dependent on a balance between androgen and estrogen action, even at the level of microtubule polymerization.

Science is like a giant jigsaw puzzle, which at first looks overwhelming, but suddenly we begin to see clues to the picture in pieces sitting on the side. It is fun to make surprising discoveries, but it can also be very difficult when trying to publish findings that go against a prevailing hypothesis. However, in the long run the surprising discoveries keep research exciting and sometimes provide the fastest leap forward. Efferent ductules of the male reproductive tract provided that excitement in my career, but also helped us to discover the importance of estrogen in the male.

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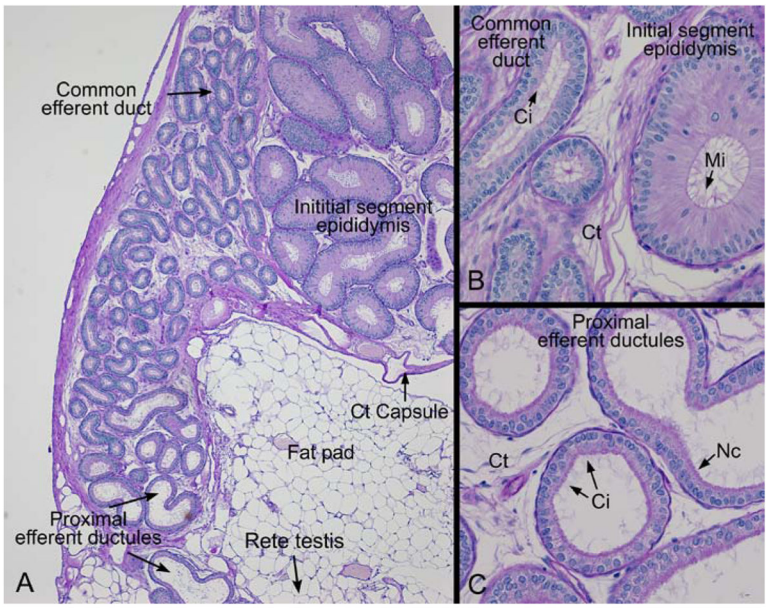

Figure 1.

Mouse efferent ductules. A. Proximal ductules have a wider lumen than the common efferent duct that is adjacent to the initial segment epididymis. Most of the ductules are surrounded by an epididymal fat pad. Location of the rete testis is indicated by the arrow. Ct, connective tissue capsule surrounding the head of the epididymis. B. Higher magnification showing the common efferent duct with a thinner epithelium than the initial segment epididymis. Ci, cilia; Mi, microvilli; Ct, connective tissue. C. Higher magnification of the Proximal efferent ductules showing cilia extending into the lumen (Ci) and nonciliated cells (Nc) with a PAS+ brush border.

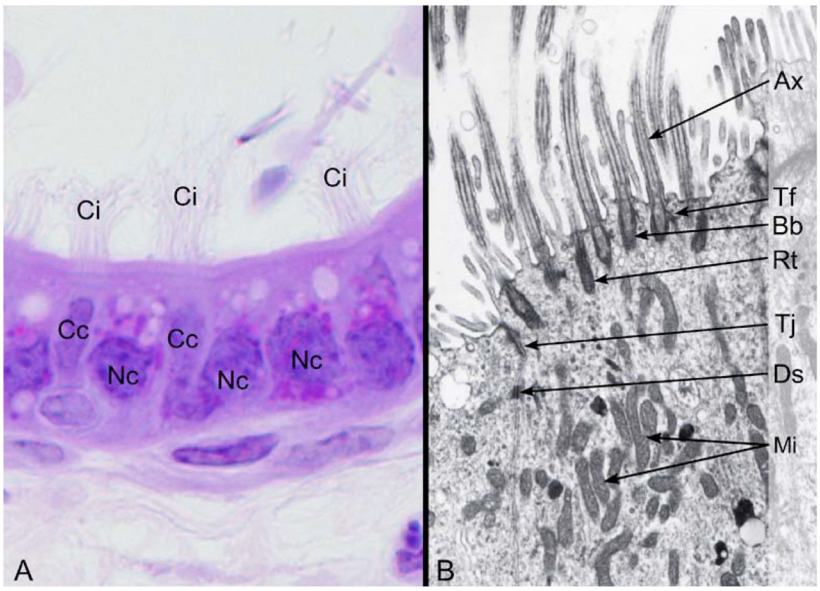

Figure 2.

Mouse efferent ductule epithelium. A. Light microscopy with PAS staining showing ciliated (Cc) and nonciliated (Nc) cells. Ciliated cell nuclei are located closer to the lumen and long cilia extend into the lumen (Ci). B. Electron microscopy of the ciliated cell. Ax, axonemal complex within a cilium; Tf, transitional fibers attached to the basal body (Bb); Rt, striated rootlet extending from the basal body into the apical cytoplasm; Tj, tight junctional complex; Ds, desmosome; Mi, mitochondria in the apical cytoplasm near the ciliary basal bodies.

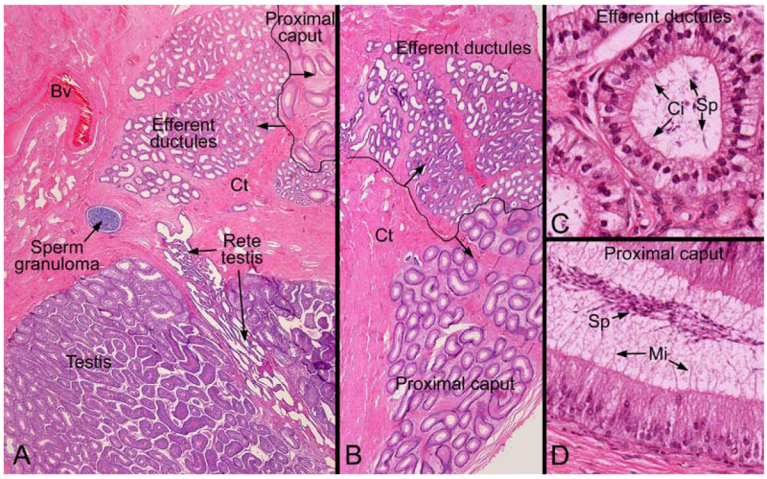

Figure 3.

Dog head of the epididymis showing its attachment to the testis by thick bundles of connective tissue (Ct). Efferent ductules are found in lobules adjacent to the proximal caput epididymis and connected to the rete testis chambers that extend into the testis. A blind ending efferent ductule that is filled with stagnant sperm forms a large sperm granuloma. Bv, blood vessel. B. Another region of the head of the epididymis showing the smaller efferent ductules separated from the proximal caput epididymis by bundles of connective tissue (Ct). C. Higher magnification of one region of the efferent ductule, showing fewer luminal sperm (Sp) than in the epididymis. Long cilia (Ci) extend into the lumen. D. Higher magnification of the proximal caput epididymis, showing higher concentration of luminal sperm (Sp). Long microvilli (Mi) extend into the lumen.

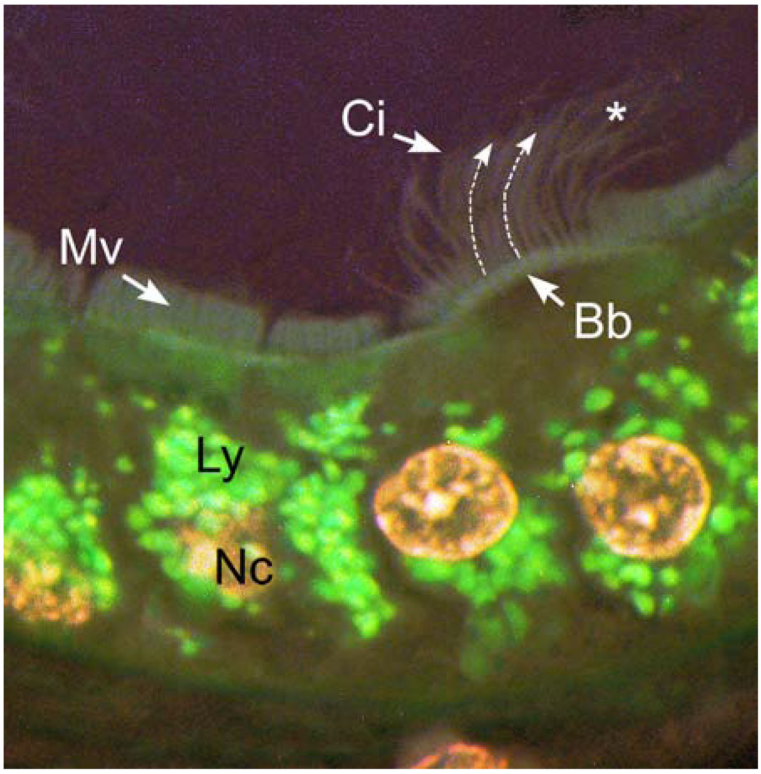

Figure 4.

High magnification light microscopy of the efferent ductule epithelium with inverted imaging to illustrate the coordinated pattern of cilia (Ci) bending to the right (white dashed arrows) from the basal body (Bb), but an irregular pattern at their tips (*). Nc, nonciliated cell; Ly, lysosomes; Mv, microvilli.

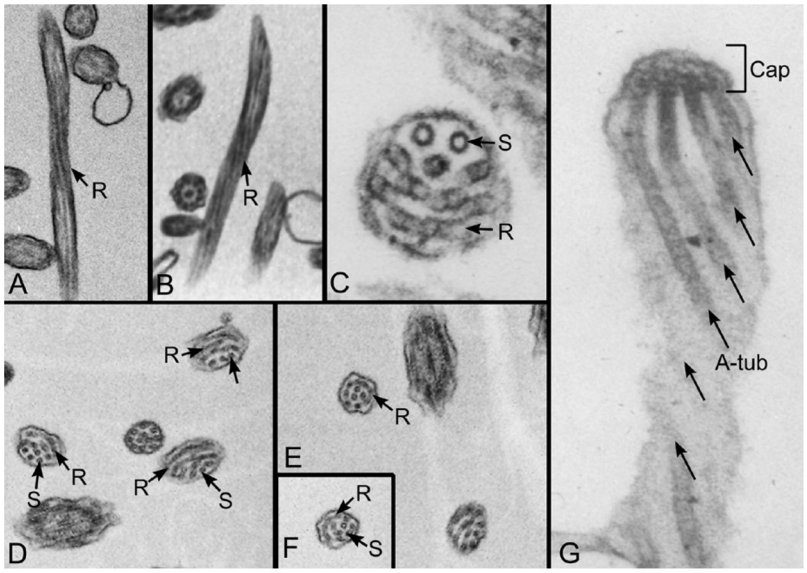

Figure 5.

Electron microscopy of efferent ductule cilia. A–B. Longitudinal sections through cilia near the tip, showing twisting of the microtubules (arrows). C–F. Cilia near the tip, showing microtubules with rotational appearance (R) adjacent to cross sections or straight microtubules (S), suggesting twisting of the cilium near the tip. G. Higher magnification of the longitudinal tip of a cilium. The cap is observed as a dense structure beneath the plasmalemma, to which the individual A-microtubules (A-tub) attach at their ends. The A-microtubules are at angles (arrows) showing the twisting of the ciliary tip.

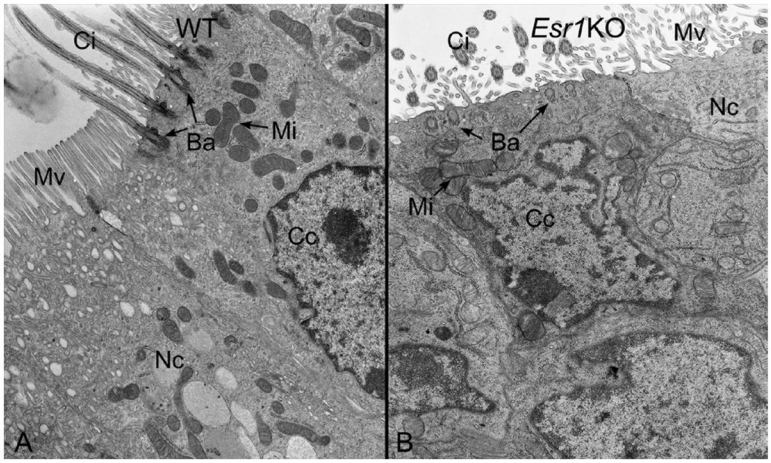

Figure 6.

Electron microscopy of ciliated cells from wild-type (WT) and estrogen receptor-α knockout mice (*Esr1*KO). Cc, ciliated cell nucleus; Nc, nonciliated cell; Mv, microvilli. A. WT efferent ductule ciliated cell showing cilia (Ci) extending from basal bodies (Ba) and mitochondria between the basal bodies and the nucleus (Cc). B. *Esr1*KO efferent ductule ciliated cell shows fewer number of cilia (Ci) and basal bodies (Ba).

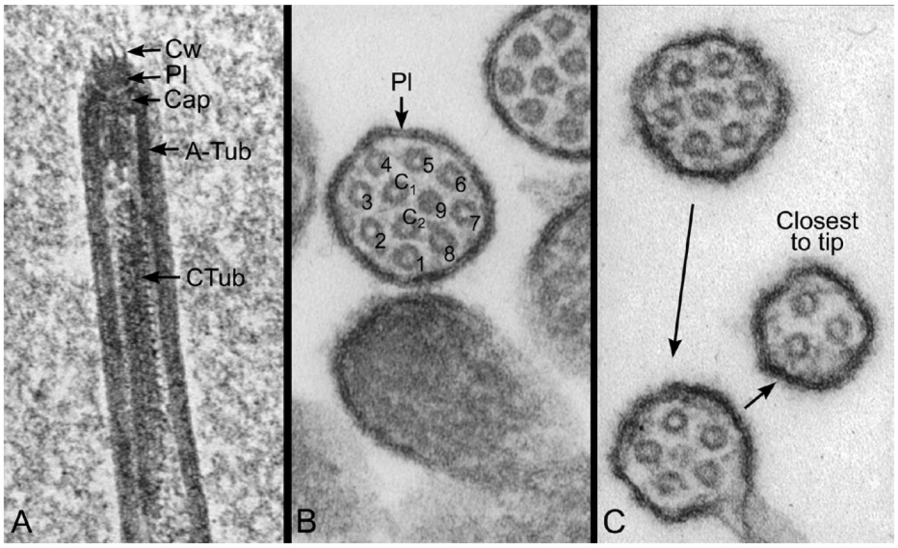

Figure 7.

Electron microscopy of efferent ductule cilia. A.Higher magnification of the longitudinal tip of a cilium. The distinct claw structure (Cw) that sits on a plate (Pl) and a cap. The A-microtubule (A-tub) is attached to the cap. Central microtubules (CTub) appear to also attach to the cap. B. Cross section through cilium near the tip, showing 9 single microtubules (arrows). The ninth microtubule joins the two central microtubules (C_1 and C_2). C. Three cross sections of cilia near the tip. The arrows show which section is the next closest to the tip, as the number of microtubule cross sections decreases at the tip.

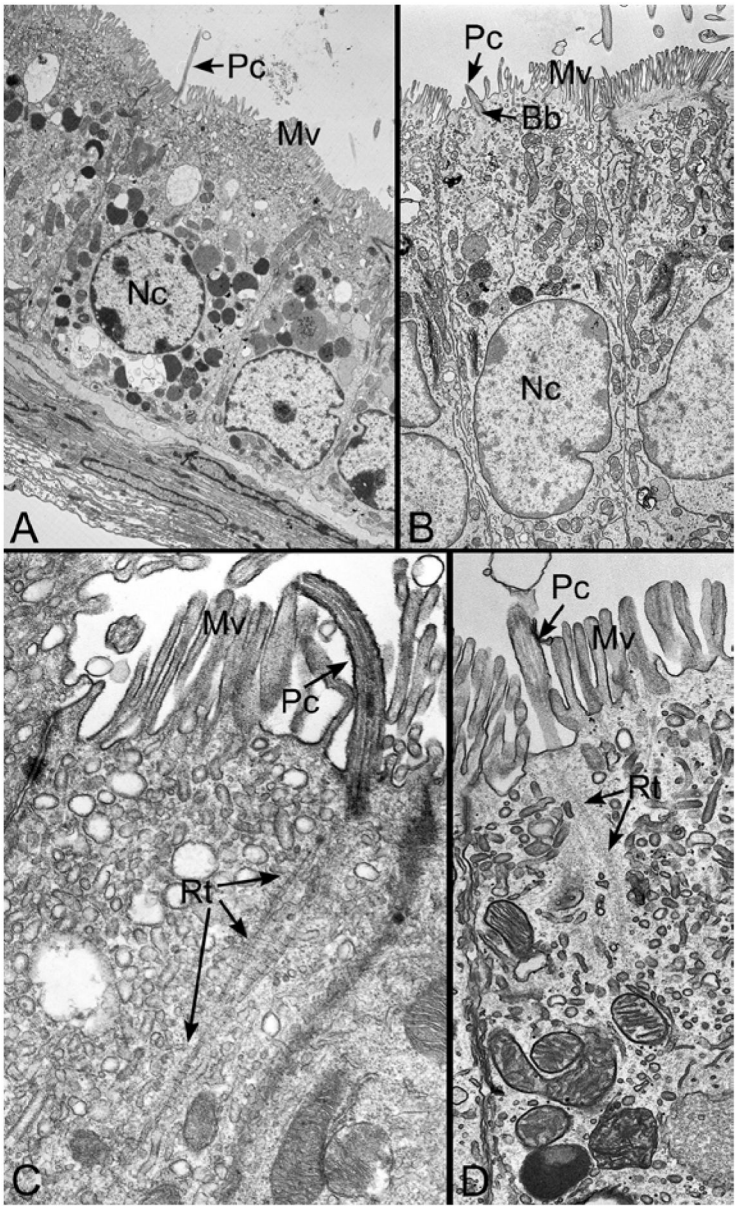

Figure 8.

Electron microscopy of nonciliated cells (Nc) from wild-type mice showing a single primary cilium (Pc) extended from the apical surface of the efferent ductule epithelium. A. The long Pc is near the tight junctional area, with microvilli (Mv) covering the remainder of the surface. B. A short Pc extends from the basal body (Bb) of the nonciliated cell. Mv, microvilli. C–D. Higher magnifications reveal long striated rootlets (Rt) extending from the basal body of the (Pc) into the apical cytoplasm. Mv, microvilli.

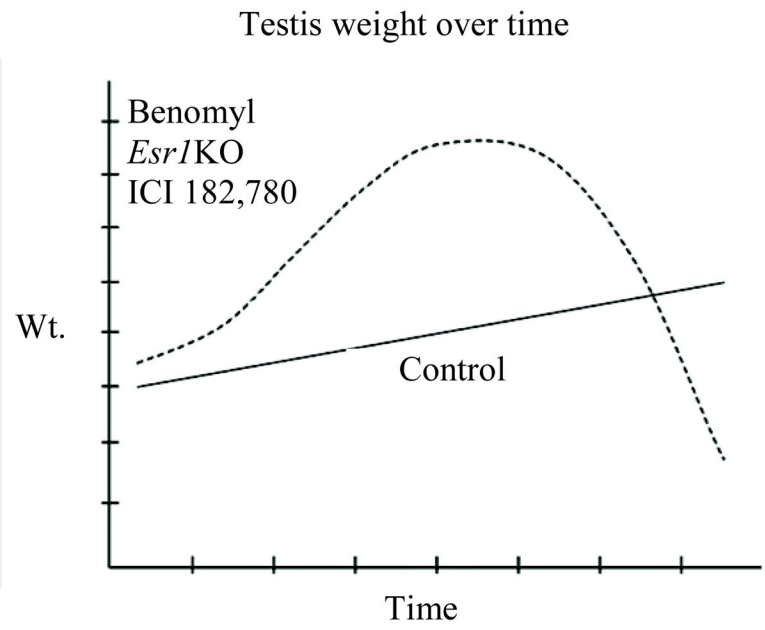

Figure 9.

A graphical depiction of testis weight changes over time in benomyl and ICI 182,780 treatment males and the estrogen receptor-α knockout mouse (*Esr1*KO). The weight increases until nearly double compared to controls and then rapidly declines with degeneration of the seminiferous epithelium until atrophy.

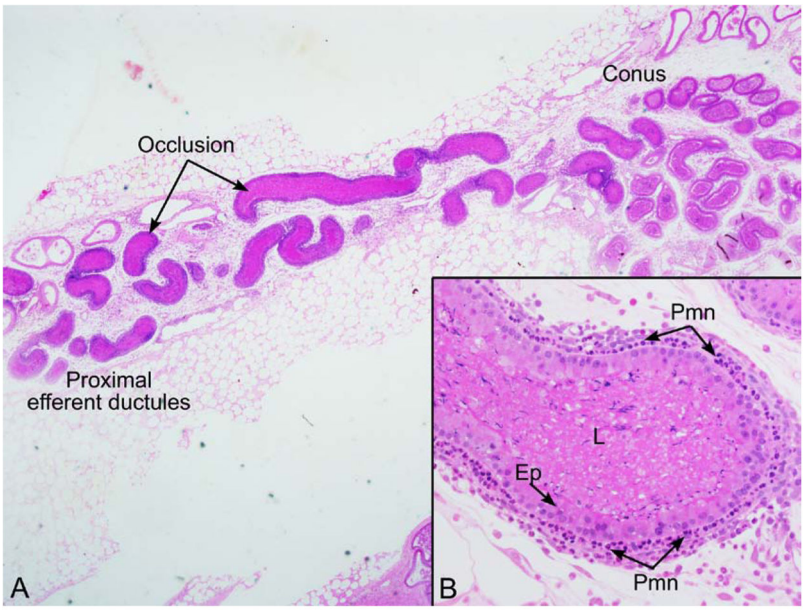

Figure 10.

A. Low magnification of occlusions of the efferent ductules following treatment with benomyl. The lumens are compacted with sperm and debris, from the proximal region to the conus. B. At higher magnification the compaction of sperm and debris from the testis seen in the lumen (L). Polymorphonuclear leukocytes (Pmn) completely surround the epithelium at 4 h post treatment.

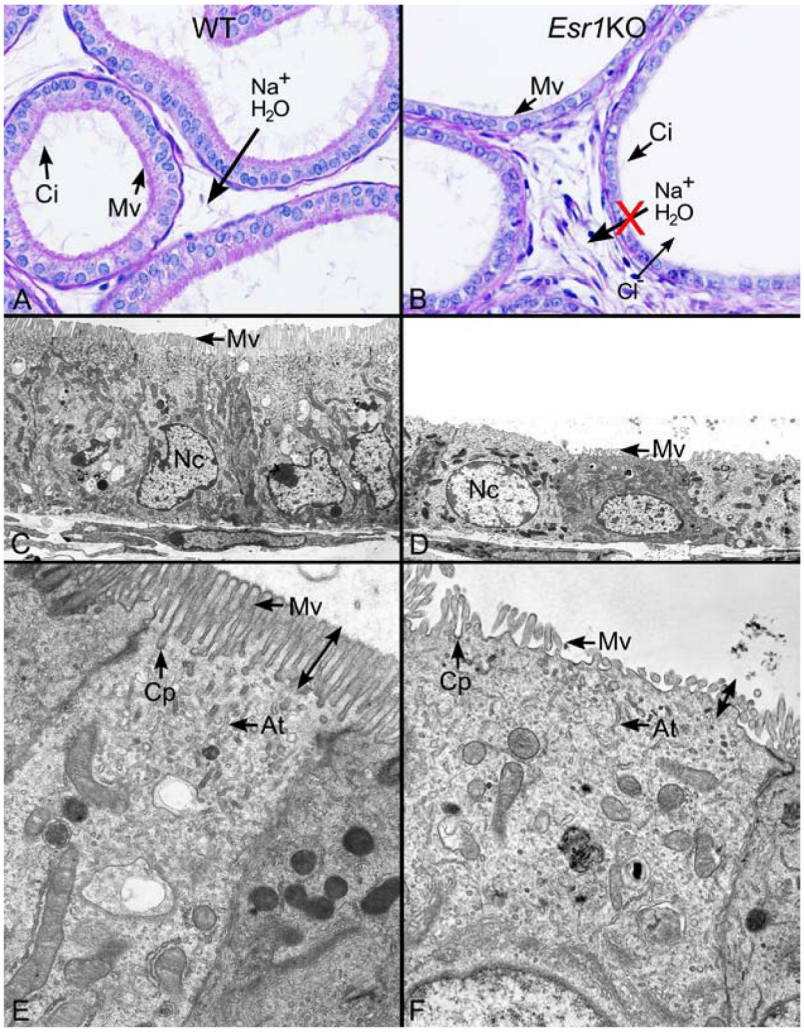

Figure 11.

Wild-type (WT) and estrogen receptor-α knockout (*Esr1*KO) efferent ductules in the proximal region. A, C, E. WT ductules showing normal cilia (Ci) extending into the lumen and a prominent brush border of microvilli (Mv) that stains with PAS. Normal physiology maintains the rapid transport of Na+ and passive movement of water (H_2O) across the epithelium. With electron microscopy, the nonciliated cell (Nc) microvillus border (Mv) is more prominent and shows coated pits extending between the microvilli and apical tubules (At) penetrating the apical cytoplasm. Height of the microvillus border (double arrow) is contrasted with that of the *Esr1*KO mouse. B, D, F. *Esr1*KO ductules have a thinner epithelium, a much wider lumen, and less prominent microvillus border (Mv) showing less PAS staining. Abnormal physiology is depicted to show the inhibition of fluid resorption by blocking Na+ transport and H_2O movement across the epithelium. An increase in Cl^- transport into the lumen is indicated. Electron microscopy shows how dramatic the decrease in epithelial height has become, with the loss of apical cytoplasm and microvilli (Mv). Height of the microvilli is greatly reduced (double arrow), as well as the number of coated pits (Cp) and apical tubules (At).

Table 1

Unique features of the efferent ductules in the male reproductive tract.

Unique features	Comments	References
1. Motile cilia	Cilia have a rotational beat that stirs the fluid	Becker, 1857; Reid and Cleland, 1957; Hess *et al.*, 2000; Hess, 2002
2. Kidney-like function	Resorb nearly 90% of the luminal fluids; transport fluid rapidly	Clulow *et al.*, 1996, 1998; Man *et al.*, 1997; Hansen *et al.*, 1999
3. Estrogen target	ESR1 is in greater concentration than in uterus; constitutively expressed	Hess *et al.*, 1997b, 2011; Joseph *et al.*, 2011

Table 2

Location and functions of mammalian cilia.

Functions	Organs	Comments	References
1. Beating or wave motion	Trachea (respiratory system); ependyma (nervous system); oviduct (female reproductive system)	Purpose is to move liquid over the surface of the epithelium in a coordinated, metachronal wave	Dalen, 1983; Satir and Christensen, 2007
2. Stirring of fluids	Efferent ductules (male reproductive system); Hensen's nodal pit (embryo)	To maintain homogeneous uptake of fluid in efferent ductules; provides left/right symmetry in development of the embryo	Brody *et al.*, 2000; Satir and Christensen, 2007; Yoshiba *et al.*, 2012
3. Grasping	Crown tip claws adhere to oocytes cumulous cells in oviduct	Help move oocyte toward ampulla for fertilization	Kuhn and Engleman, 1978; Dalen, 1983; Portman *et al.*, 1987; Kubo *et al.*, 2008; Fisch and Dupuis-Williams, 2011
4. Vibration	Tethering of otolith stones for inner ear development	Unique to the inner ear	Colantonio *et al.*, 2009; Stooke-Vaughan *et al.*, 2012
5. Sensory signaling	Chemical receptors, coordination of beat; mechanosensory activity	Olfactory cilia are immotile; nearly all cells have a single primary cilium that is sensory and provides symmetry	Verghese *et al.*, 2008; Veland *et al.*, 2009; Satir, 2010; Satir *et al.*, 2010; Wilson and Stainier, 2010; Bloodgood, 2012; Knowles *et al.*, 2013

Table 3

Possible explanations for the aromatase knockout (ArKO) mouse phenotype.

1	ESR1 is constitutively expressed in efferent ductule epithelium in the absence of estrogen (Oliveira *et al.*, 2004; Toda *et al.*, 2008).
2	ESR1 is expressed in efferent ductules of the ArKO mouse (Toda *et al.*, 2008); therefore, ESR1 may activate a ligand-independent pathway (Power *et al.*, 1991; O'Malley, 2005; McDevitt *et al.*, 2007, 2008; Sinkevicius *et al.*, 2008, 2009).
3	ESR1 could bind a metabolite of dihydrotestosterone or other steroids that are present in high concentrations in the male (Picciarelli-Lima *et al.*, 2006).
4	Maternal estrogens during development may be sufficient to permit normal development of rete testis and efferent ductules in the ArKO and dietary phytoestrogens may be sufficient to activate ESR1-mediated pathways in the adult males (Robertson *et al.*, 2002); dietary phytoestrogens may be 'agonistic' in the absence of endogenous estrogen but 'antagonistic' when endogenous estrogens are present (Ruz *et al.*, 2006).
5	Treatment with an aromatase inhibitor also showed no effect on efferent ductule morphology, but did decrease the expression of ESR2 and GPER, while increasing androgen receptor (Pearl *et al.*, 2007; Lazari *et al.*, 2009; Gomes *et al.*, 2011); this combination may activate fluid resorption through androgen-response elements of the *Slc9a3* gene (Zhou *et al.*, 2001).
6	Estrogen function in the male reproductive tract may be a combination of receptor-mediated transcriptional activity, both ligand dependent and independent, as well as rapid effects through a membrane receptor (Revankar *et al.*, 2005; Carreau *et al.*, 2006; Filardo *et al.*, 2007; McDevitt *et al.*, 2007, 2008; Lucas *et al.*, 2008, 2010; Sinkevicius *et al.*, 2008, 2009; Weiss *et al.*, 2008; Lazari *et al.*, 2009; Carreau and Hess, 2010; Hess *et al.*, 2011).