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Disruption of estrogen receptor signaling and similar pathways in the efferent ductules and initial segment of the epididymis

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Seminiferous tubular atrophy may involve indirectly the disruption of estrogen receptor-α (ESR1) function in efferent ductules of the testis. ESR1 helps to maintain fluid resorption by the ductal epithelium and the inhibition or stimulation of this activity in rodent species will lead to fluid accumulation in the lumen. If not resolved, the abnormal buildup of fluid in the head of the epididymis and efferent ductules becomes a serious problem for the testis, as it leads to an increase in testis weight, tubular dilation and seminiferous epithelial degeneration, as well as testicular atrophy. The same sequence of pathogenesis occurs if the efferent ductule lumen becomes occluded. This review provides an introduction to the role of estrogen in the male reproductive tract but focuses on the various overlapping mechanisms that could induce efferent ductule dysfunction and fluid backpressure histopathology. Although efferent ductules are difficult to find, their inclusion in routine histological evaluations is recommended, as morphological images of these delicate tubules may be essential for understanding the mechanism of testicular injury, especially if dilations are observed in the rete testis and/or seminiferous tubules.

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The rete testis and efferent ductules can appear dilated, as if the lumens were greatly expanded with excess fluid or the accumulation of sperm. Because the efferent ductules resorb most of the fluid arriving from the rete testis lumen, one of two mechanisms is likely to be involved: a) reduced fluid uptake, which has been caused by the disruption in estrogen receptor signaling or associated pathways; or b) an increased rate of fluid resorption, which results in luminal occlusion. Both mechanisms can lead to a temporary increase in testicular weight, tubular dilation and atrophy of the seminiferous tubules.

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Introduction

Testicular atrophy is one of the more easily recognized endpoints in male reproductive pathology; however, an interpretation of the mechanism causing seminiferous tubular atrophy is not always easy to uncover. The observation of luminal dilation in the rete testis and/or seminiferous tubules is a signature lesion that could lead one to conclude that testicular atrophy may be a long-term outcome. It has been known since 1924 that occlusion of the efferent ductules near the rete testis will induce increased pressure within the seminiferous tubules and lead to testicular atrophy.[1] Yet, the literature is filled with long-term studies showing testicular atrophy, without histopathological evaluation of the efferent ductule region. This is partially due to the difficulty in finding these delicate tubules that are buried in the epididymal fat pad of rodents,[2] but also because for years most authors considered these ducts to be nothing more than a conduit from rete testis to the epididymis.[3] However, evidence began to reveal that disruption of the kidney-like function of efferent ductules could result in fluid accumulation within the rete testis and seminiferous tubules and eventually testicular atrophy.[4,5] One of the disrupting pathways uncovered was estrogen receptor-α (ESR1).[6]

As early as the 1930's, it was known that developmental exposure to high doses of natural estrogens, as well as diethylstilbestrol (DES) could induce malformation of the male reproductive tract.[7-9] However, the prevailing hypothesis to explain these data was that estrogen exposure disrupted testosterone and its metabolite 5α-dihydrotestosterone (DHT), the dominant male sex steroid[10] and that estrogen did not have a distinct function in the adult male reproductive tract, but rather played a role in early development during the ambisexual stage and in establishing male behavioral patterns.[11] In 1997, examination of the estrogen receptor α knockout mouse (*Esr1*KO) revealed that ESR1 has a major function in regulating fluid resorption in efferent ductules of the testis,[6] which is essential for increasing the concentration of sperm and their maturational development in the head of the epididymis.[12-14]

Efferent ductules are small, coiled tubules that transport sperm rapidly from rete testis chambers to the epididymal head (**Fig. 1**). In rodent species, efferent ducts are buried in the epididymal fat pad, beginning as 3-7 individual wide-lumen ducts but merging into a single, highly convoluted tubule with a narrow

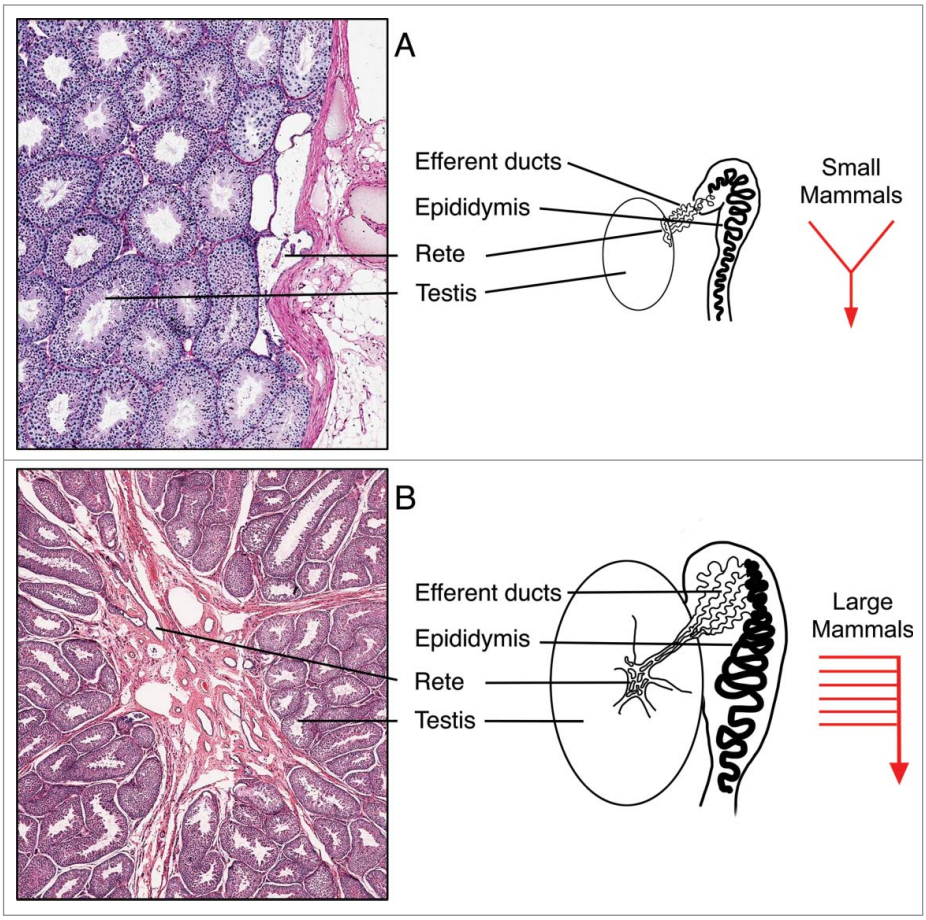

Figure 1. Basic organizational patterns of the rete testis and efferent ductules in small and large mammals. (**A**) In smaller mammals, such as rats and mice, the rete testis forms flattened chambers adjacent to the tunica albuginea of the testis, where sperm and tubular fluids are released into 3-7 efferent ductules that merge to form a single, highly convoluted common duct that enters the initial segment epididymis. (**B**) In larger mammals, including dogs and man, the rete testis forms flattened chambers surrounded by dense connective tissue within the mediastinum of the testis, which drains toward the efferent ductules that occupy a major portion of the caput epididymis. Most of the efferent ductules open individually into the caput epididymis.

lumen under the capsule of the initial segment of the epididymis.[5] In man and larger mammals, these ductules are more numerous than in rodent species and open independently into the epididymis at multiple sites in the caput epididymis. Most importantly, these ductules form the major portion of the caput region within a densely organized connective tissue that is attached to the tunica albuginea of the testis.[5] The discovery that ESR1 is essential for male fertility altered our view of the role that efferent ductules play in the head of the epididymis and provided the basis for testing new hypotheses to explain numerous observed pathologies in the testis and epididymis.[3,4,15-18] Several reviews have been written about estrogen's function in the male reproductive tract and should be examined for a more detailed understanding of its molecular interactions and physiological relevance.[12-14,19,20] However, histopathological changes in testis and epididymis following ESR1 disruption were found to be similar to those observed after exposures to several environmental compounds and some classes of therapeutic biological products, as well as surgical ligation of the ductules. Therefore, this review will focus on some common histopathological responses of the efferent ductules and head of the epididymis that induce fluid accumulation in the testis, which may contribute to the atrophy of seminiferous tubules.

Source of Estrogen in the Male Reproductive Tract

Estrogen synthesis is controlled by the aromatase enzyme complex of cytochrome P450 (P450arom) encoded by the *CYP19* gene and a ubiquitous NADPH cytochrome P450 reductase.[21] Testis is a major site for estrogen synthesis in the male and for many years it was assumed that Sertoli cells were the primary source during development, but in the adult only Leydig cells produced estrogen.[22] Immunolocalization of P450arom was a major challenge, but in 1993 Nitta et al.[23] became the first laboratory to demonstrate its presence in the mammalian spermatid (**Fig. 2**) and cytoplasmic droplets of sperm traversing the epididymis.[24,25] The high concentrations of systemic androgens throughout the body are a blunt force on nearly every tissue in the male, but the unique system of estrogen synthesis in the male reproductive system creates a sequestered androgen/estrogen balance that can be focused specifically on cells expressing the requisite steroid receptors.

It was surprising that the P450arom knockout mouse (AromKO) did not show histopathological results[26-29] similar to the *Esr1*KO mouse.[6,12] Testicular degeneration in the AromKO male began with ageing and was independent of the efferent ductule abnormalities found in the *Esr1*KO. Several explanations have been proposed and some have been tested. First, ESR1 expression in the efferent ductule epithelium is constitutive and thus continues to be expressed in the absence of natural ligand[29,30] and could be activated in a ligand-independent manner.[31-33] It is also possible that an ever-present ESR1, in the absence of estradiol, could bind a metabolite of DHT or other steroids that are present in high concentrations in the male.[34] Finally, dietary phytoestrogens have also been shown to be sufficient for activation of *Esr1*-mediated pathways in the AromKO male[28] and to increase the concentration of cauda epididymal sperm in Wild-type and *Esr1*KO mice[35] It has been suggested that dietary phytoestrogens may be 'agonistic' in the absence of

endogenous estrogen but 'antagonistic' when endogenous estrogens are present.[12] Treatment with an aromatase inhibitor[36-38] also showed no effect on efferent ductule morphology, but did decrease the expression of ESR2 and GPER, while increasing androgen receptor (AR) in the rat. It also delayed the development of the head of the epididymis. Thus, disruption of estrogen pathways in the male can lead to subtle or delayed histopathological results and depend on the presence or absence of its receptors, which are constitutively expressed in efferent ductules.[29,30]

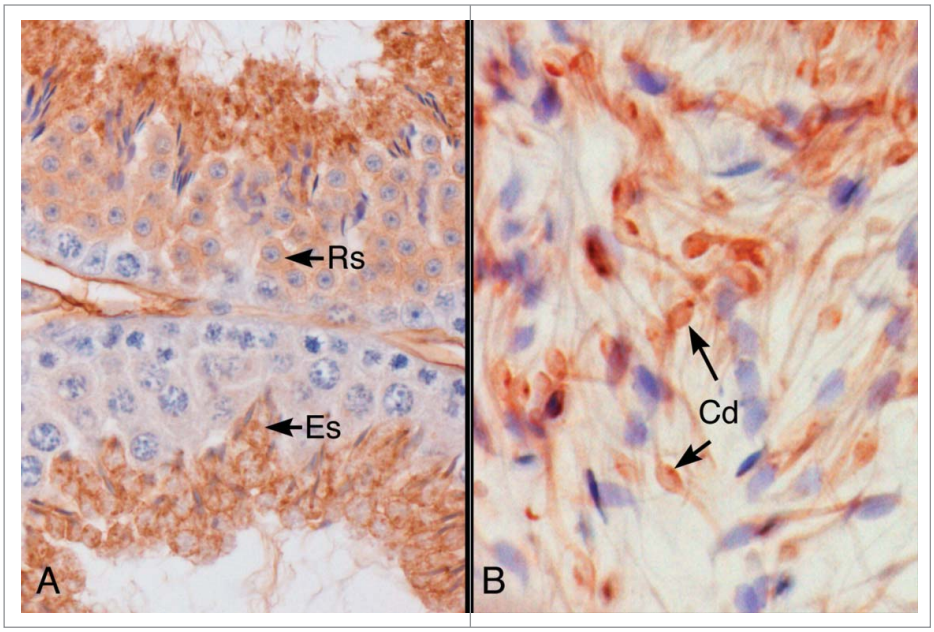

Figure 2. Immunohistochemical localization of P450 aromatase protein in the mouse testis and epididymis. (**A**) Aromatase protein was localized in the cytoplasm of round (RS) and elongated spermatids (ES) in the mouse seminiferous epithelium. (**B**) Caput epididymal lumen. Aromatase protein was localized in the cytoplasmic droplet (Cd) and along the thin tails of the spermatozoa.

Estrogen Receptors in the Male

The presence of the female hormone in the male reproductive tract suggested that the target cell and tissue for this luminal estrogen could be the epididymal epithelium, luminal sperm or even the female reproductive tract. Classical mediation of estrogen function is through two estrogen receptors, ERα (ESR1) and ERβ (ESR2), which are members of the nuclear receptor family of transcription factors and bind to estrogen response elements to mediate gene transcription.[39-41] It has been known for 35 years that an estrogen receptor-like protein exists in male reproductive tissues[42] and that estradiol binding is very strong in efferent ductules and the initial segment epididymis.[43] Subsequent studies confirmed this hypothesis, as the efferent ductules were found to express *Esr1* mRNA 3.5-fold greater than uterine tissue[44] and immunohistochemistry[13,14,19] revealed intense co-localization of ESR1 and AR in both ciliated and non-ciliated cells of the epithelium (**Fig. 3**).

In contrast, localization of ESR1 in the testis and epididymis has been a challenge, as major differences are found between species, as well as between individuals within a species. Results differ between immunohistochemical localization and mRNA analysis of testicular tissues and depend upon antibody source, age of development and experimental design.[12-14,16,19,45-52] In general, most studies have concluded that testicular expression of ESR1 is low, but under certain conditions and in some species can be found in germ cells of the testis and sperm.[12] On the other hand, ESR2 is expressed nearly ubiquitously throughout the male reproductive system.[12,47] Therefore caution must be exercised when studying estrogen action in the testis. ESR1 expression in epididymis is also controversial, due to some studies showing no immunohistochemical staining while others using better fixation and optimal staining have found the protein both in cytoplasm and the nucleus.[13]

In addition to the genomic effects of estrogen, rapid non-genomic and membrane-associated responses have finally been recognized as indisputable pathways contributing to estrogen's role in specific cellular functions, including the male reproductive system.[50,53-59] ESR1 and ESR2 are involved in rapid, non-genomic transduction effects of estradiol, but the G protein-coupled estrogen receptor-1 (GPER-1) also mediates multiple downstream signaling pathways.[59-67] However, this area of investigation has become complicated because some studies have shown an ER antagonist inhibiting GPER-1 activity,[68] while other studies show activation.[69,70]

Histopathology of Estrogen Receptor Dysfunction in Efferent ductules and Epididymis

Our acceptance of estrogen and its receptor, ESR1, having a major role in regulating fluid physiology in the male reproductive tract began with the analysis of the *Esr1* knockout mice[6,29,35,71-87] and treatment of rodents and other species with the pure anti-estrogen ICI 182,780 (ICI).[6,37,46,51,58,67-69,75,80,85,88-116] Deletion of *Esr1* gene caused male infertility, not only due to a disruption in male sexual behavior,[31] but also because the sperm failed to mature properly in the male reproductive tract.[117,118] Treatment with ICI induced subfertility at first, but over time complete infertility[88,89] and resulted in numerous histopathological changes that were similar to those found in testes, efferent ductules and epididymides of the *Esr1*KO.

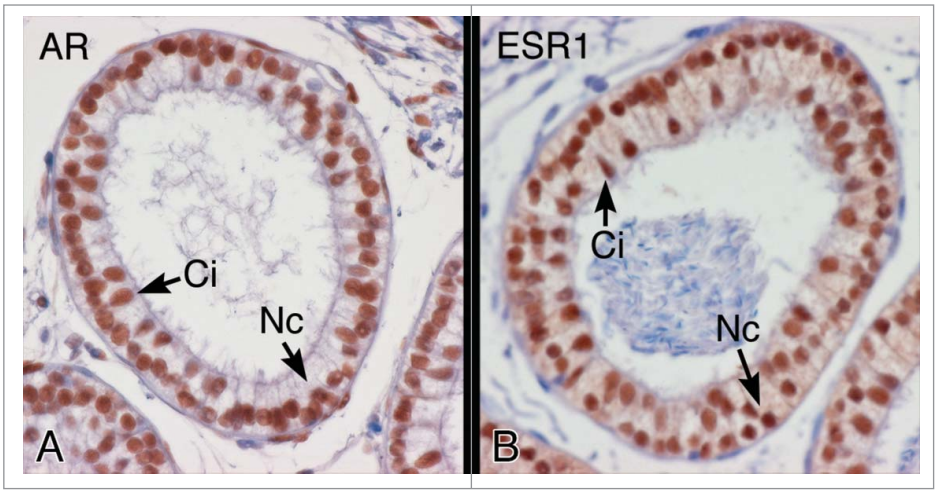

Figure 3. Androgen receptor (AR) and estrogen receptor-α (ESR1) protein in the efferent ductule epithelium of the hamster. (**A**) AR protein shows intense nuclear staining in both ciliated (Ci) and nonciliated (Nc) cells of the proximal efferent ductule epithelium. (**B**) ESR1 protein also shows intense nuclear staining in ciliated (Ci) and nonciliated (Nc) cells of the proximal efferent ductule epithelium.

There are two basic mechanisms known to cause fluid accumulation and backpressure atrophy of the testis (**Fig. 4**): a) Inhibition of fluid resorption by the efferent ductule epithelium causing luminal dilution, and b) Compaction of the luminal contents causing occlusion of the efferent ductules. ESR1 disruption (**Table 1**) involves the first mechanism in rodent species because rodent efferent ductules have essentially a funnel-like design (**Fig. 1**). When the accumulation of luminal fluids exceeds the capacity of the single common duct exit, fluid pushes back into the testis causing dilation of rete testis and the seminiferous tubules.[13,14,19]

Histopathological changes in the male reproductive system following Esr1 disruption were consistent with the inhibition of fluid reabsorption by the efferent ductule epithelium.[6] Severe dilation of the lumen (**Fig. 5**) was observed in the efferent ductules, rete testis and seminiferous tubules.[6,29,35,71,74,76,78,79,86] Estrogen action through ESR1 regulates directly a number of major genes or indirectly several proteins involved in ion exchange and water transport in the efferent ductule epithelium. Most notably, ESR1 helps to maintain the activity of sodium/hydrogen exchanger-3 (SLC9A3) and aquaporins 1 and 9 (AQP1, AQP9), which facilitate the resorption of Na+ and water. Also ESR1 provides an inhibitory influence on the Cl- transporters cystic fibrosis transmembrane conductance regulator (CFTR) and Slc26a3 (DAR), as well as Na+/K+ ATPase α1 (Slc9a1), which would decrease the secretion of Cl- and movement of water at the luminal surface, while balancing the removal of cytoplasmic Na+ at the basal plasmalemma. Fluid resorption is further dependent on the endocytic apparatus of the nonciliated cells,[119] which was also disorganized after the disruption of ESR1 activity.[6,76,88,89,95]

Recent studies have shown that estrogen works through the classical activation function (AF) domain, AF-1, but is regulated by the AF-2 domain.[97] However, it also maintains a capability for ligand-independent activation in the efferent ductule epithelium, possibly working through phosphorylation of the AF-1 domain,[120] or even its membrane receptor.[13,50,53-59] Disruption of this ESR1 activity alters the luminal fluid composition, resulting in an alkaline, hypo-osmotic environment that resulted in abnormal sperm morphology.[117,121] Treatment of the *Esr1*KO sperm with cAMP rescued all defective motility parameters.

In addition to the fluid-transport genes, estrogen also regulates several structural proteins responsible for maintenance of the efferent ductule epithelium. Loss of ESR1 activity resulted in significant alterations in epithelial morphology (**Fig. 6**). There was a 52% reduction in epithelial height, decreases in the endocytic apparatus, a dramatic reduction in the number and size of microvilli and also cilia.[6,29,37,47,74-76,89,90,122] Thus, both direct effects (those regulating proteins necessary for ions and water fluxes) and indirect (epithelial morphology) were mediated by ESR1 inactivation in this critical region of the male tract.

Several other gene manipulation models and chemical treatments (**Table 1**) also inhibit fluid resorption in the efferent ductules, resulting in dilation of rete testis and seminiferous tubules. However, many of these appear to either decrease ESR1 activity[97,123-125] or inhibit ESR1 associated pathways.[77,126,127] Surprisingly, the knockout of two genes regulated by ESR1, *Slc9a3* and *Car2*, produced normal epithelial morphology in the efferent ductules, while exhibiting luminal dilations of the ductules and rete testes that were greater than those observed in the *Esr1*KO.[77] Thus, from a histopathological viewpoint, fluid accumulation with luminal dilation may or may not be associated with altered epithelial morphology. One explanation might be that efferent ductules adapt to the accumulation of fluid in the *Slc9a3* and *Car2* knockout mice and simply show excessive growth during development. When evaluating global gene knockout mice, it becomes difficult to separate developmental versus adult functions of a gene. This problem was seen in *Esr1*KO model, as the rete testis and efferent ductules were are already dilated at 10 days of age, prior to puberty.[78] Therefore, treatment of the adult male with the antiestrogen ICI was necessary to show ESR1 regulation of both epithelial morphology and physiological function, separate from any developmental influence.

Finally, understanding estrogen activity in the epididymis has been a challenge because androgens have the primary role in its

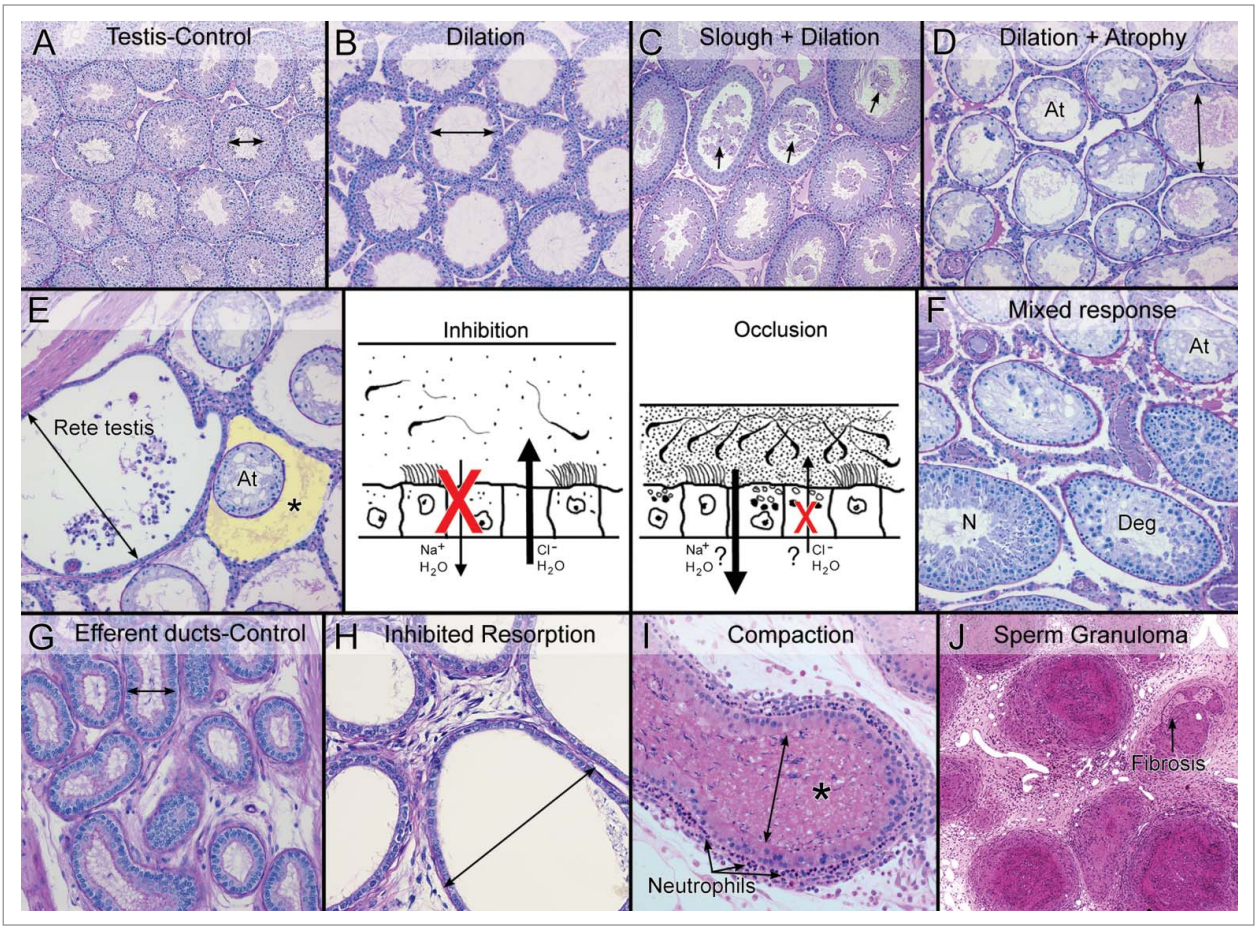

Figure 4. Two mechanisms lead to efferent ductule dysfunction and fluid accumulation in the testis. The central drawing illustrates the two mechanism of efferent ductule dysfunction that will result in the accumulation of luminal fluids and cause backpressure damage to the seminiferous tubules. The 'inhibition' mechanism involves the blockage of fluid resorption by inhibiting Na+ and water update and possibly an increase in Cl- and water movement into the lumen, thereby diluting the sperm and exceeding the drainage capacity of the ductules into the epididymis. The 'occlusion' mechanism involves excessive resorption and possibly an inhibition of Cl- secretion into the lumen. This mechanism results in a more viscous luminal environment, sperm stasis and eventually the occlusion or blockage of the ductule. (**A**) Control testis showing normal cross-sectional widths of the seminiferous tubular lumens. (**B**) Testis showing dilation of the seminiferous tubular lumen caused by the inhibition mechanism. Spermatogenesis appeared normal but there was thinning of the epithelium. (**C**) Testis showing dilation of the tubules caused by the occlusion mechanism. Sloughing of germ cells into the lumen (arrows) was also involved. (**D**) Testis showing seminiferous tubular atrophy (At), with some evidence of residual dilation after long-term occlusion of the efferent ductules. (**E**) Rete testis region showing excessive buildup of fluid and dilation, adjacent to atrophic (At) seminiferous tubules following the inhibition of fluid resorption. The yellow highlighted area (*) illustrates an edematous buildup around the atrophic tubules, which occurs in some cases but not in others. (**F**) Testis showing a mixed response following long-term occlusion of the efferent ductules. Atrophic tubules (At) are mixed with normal spermatogenesis (N) and degenerative changes (Deg). (**G**) Control efferent ductules in the conus region showing a normally narrow luminal diameter. (**H**) Efferent ductules at the proximal/conus junction following the inhibition of fluid resorption show excessive dilation and thinning of the epithelium. (**I**) Compaction of sperm within the lumen of the efferent ductules leads to dilation of the lumen and occlusion. This response caused the recruitment of polymorphonuclear leukocytes (neutrophils) into the wall lining the epithelium. (**J**) A long-term consequence of efferent ductule occlusion is the formation of sperm granulomas. The hyaline area shows the beginning of fibrosis.

regulation.[128] Historically, others have used castration followed by estrogen treatment models to study estrogen function in the epididymis. However, such studies must now be reinterpreted because ESR1 is constitutively expressed in efferent ductules after castration and high dosages of estradiol down-regulated both AR and ESR1.[30,91] Thus, an interpretation of the castration model as being representative of estrogen's function in the epididymis appears to be invalid.

Histopathology of Occlusions in Efferent Ductules and the Epididymal Head

Compaction of the luminal contents with occlusion of the efferent ductules is the second basic mechanism known to cause fluid accumulation and backpressure atrophy of seminiferous tubules (**Fig. 4**). If sperm production and Sertoli cell secretions continue uninhibited following efferent ductule blockage, the

Table 1. Causes of efferent ductule dysfunction, with potential for the induction of testicular atrophy

CAUSE	DESCRIPTION	POTENTIAL TARGET[a]	REFERENCES
CHEMICAL			
ICI 182,780	Fulvestrant	Inhibition of fluid resorption; blocks ESR1 and ESR2; similar to *Esr1*KO	6,76, 78, 88-90
GR40370X	5-hydroxytryptamine receptor agonist; Serotonin-like, monoamine neurotransmitter	Inhibition of fluid resorption; vasoconstriction of venous plexus	156
PDE4 inhibitor	Phosphodiesterase-4 inhibitor	Inhibition of fluid resorption followed by occlusion; sperm granulomas	147
Uranyl nitrate hexahydrate	Dietary long-term exposure; proximal convoluted tubules of kidney sensitive	Inhibition of fluid resorption; progressive dilation of seminiferous tubules	157
LTI-1	Leukotriene A(4) hydrolase inhibitor	Occlusion; dysregulation in fluid reabsorption; sperm granuloma	2
6-chloro-6-deoxysugars	α-chlorohydrin-like chemicals	Occlusion; dysregulation of fluid resorption; sperm granuloma in efferent ductules; initial segment epididymis necrosis; inhibit glyceraldehyde-3-phosphate dehydrogenase	148,158-165
Isoproterenol	Beta-adrenergic agonist	Potential increase in rate of resorption; upregulates endothelin receptor-A; Et-1 increases Slc9a3 and inflammation	166-168
Benomyl[b]	Methyl [1-[(butylamino)carbonyl]-1H-benzimidazol-2-yl]carbamate	Occlusion; microtubule disruption; germ cell sloughing; sperm granuloma	4, 130, 131, 135, 136, 169, 170
2-Methylimidazole	Polymerization cross-linking and catalytic curing agent for epoxy resins	Occlusion; efferent duct sperm granuloma near caput epididymis	171
EDS	Ethane-1,2-dimethyl-sulfonate	Occlusion; alkylating agent, cellular toxicity; sperm granuloma	158, 172
Cadmium	Chemical element, Cd	Occlusion; vascular endothelium; sperm granuloma	173, 174
1,3-dinitrobenzene	*m*-Dinitrobenzene	Occlusion; impaired oxygen transport; sperm granuloma	175, 176
Dibutyl phthalate (DBP)	Di-n-butyl phthalate	Occlusion; prenatal exposure; epididymal malformation	177
Linuron	*N* - (3,4-dichlorophenyl)-N′-methoxy-N′-methylurea	Occlusion; herbicide; prenatal exposure; epididymal malformation	178
DES	Diethylstilbestrol	Neonatal exposure; decreases androgen receptor; sperm granuloma; dilation of lumen	179-185
Estradiol	β-estradiol 17-cypionate; 17β-estradiol; estradiol benzoate; ethinyl estradiol	Neonatal exposure; sperm granuloma; dilation lumen	181, 182, 186-188
GENE MANIPULATION[d]			
Esr1 KO	Estrogen receptor-α	Inhibition of fluid resorption; decreases in SLC9A3, CA2, AQP-1, AQP-9, CAR14, SLC4A4; increases in CFTR, SLC9A1, SLC26A3	6, 19, 35, 47, 74, 75, 77-80, 97
AF2ERKI MT	ESR1 AF-2 mutation	Inhibition of fluid resorption; blocks ESR1 AF-2 domain; similar to Esr1 KO	97
Slc9a3 KO	Sodium/hydrogen exchanger-3	Inhibition of fluid resorption	77
Car2 MT	Carbonic anhydrase II	Inhibition of fluid resorption	77
Gpr64 KO	G protein-coupled receptor 64 (He6)	Inhibition of fluid resorption	126
He6 KO	GPR64; orphan member of the LNB-7TM (B (2)) subfamily of G-protein-coupled receptors	Inhibition of fluid resorption; proximal efferent ductules; partial sperm stasis	126
Lgr4 KO or MT	G protein-coupled receptor	Inhibition of fluid resorption; decreased expression of ESR1 and SLC9A3; also occlusion	123, 124
Prkar1a+/−	Protein kinase A (PKA) type Iα regulatory subunit (RIα)	Inhibition of fluid resorption; inhibition of Slc9a3 by over phosphorylation	127
Fst OE	Follistatin; inhibitor of activin	Inhibition of fluid resorption or ductule contraction; sperm stasis; decreased expression of ESR1	125
Lfng KO	O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	[c]Notch signaling; blocked connection with efferent ducts	189
Notch1 OE	Notch homolog 1, translocation-associated	[c]Transmembrane, oncogene, efferent ductule overgrowth	190

(*continued on next page*)

Table 1. Causes of efferent ductule dysfunction, with potential for the induction of testicular atrophy *(Continued)*

CAUSE	DESCRIPTION	POTENTIAL TARGET[a]	REFERENCES
Pkd1 KO	Polycystic kidney disease 1 homolog	Abnormal epididymal development; dilation of efferent ductules	191
TE rat MT	Outbred Wistar strain	Autoimmune disorder; sperm granuloma	192
Dax1 KO	Nr0b1; transcription	[c]Occlusion; overgrowth of Sertoli cell and efferent duct epithelium	193
ProxE-AR or CEAR KO	Androgen receptor knockout in initial segment or caput epididymis	Occlusion; differentiation failure in caput epididymis; sperm granuloma	194, 195
Dicer1KO	Endoribonuclease; RNA interference	Occlusion; abnormal growth and blockage	196
HUMAN DISEASE			
Von Hippel-Lindau disease	Papillary cystadenoma of the epididymis; also cystic kidney	Dysregulation of HIF1α; upregulation of vascular endothelial growth factor (VEGF)	197-201
Young's syndrome	Chronic sinopulmonary infections; azoospermia	Abnormal secretion or resorption; occlusion of caput and middle epididymis	202-205
Varicocele	Dilation of veins near rete testis and efferent ductules	Occlusion; compression of excurrent ducts and edema; blockage	206
Spontaneous granuloma	Caput epididymis efferent ductules	Occlusion; sperm granuloma; fibrosis; recanalization	207-209
Renal failure	Renal dialysis; renal malformations; renal cysts	Dilation of rete testis and epididymis; can lead to occlusion; intraductal calcium oxalate deposits	210-216
PHYSICAL			
Ligation of ductules	Surgical blockage	Fluid accumulation; greater testicular effects when occluded closer to the rete testis	1, 129, 133, 150, 151, 217-225
Arterial occlusion	Superior epididymal artery	Occlusion; localized ischaemia, sperm granuloma	151, 226, 227

[a]Potential target for mechanisms in efferent ductules and rete testis, not necessarily testis or other organs.
[b]Including its metabolite carbendazim.
[c]Occlusion involves overgrowth of epithelium in rete testis and efferent ductules, but may also involve disruption of fluid reabsorption.
[d]Gene knockout (KO); overexpression (OE); mutation (MT).

following sequence of events will occur: a) proximal efferent ductules dilate and attempt to resorb the excess fluid; b) sperm become more compacted as fluid is resorbed; c) the rete testis begins to dilate and press into the testicular parenchyma; d) dilation of the seminiferous tubular lumens begins in regions proximal to the rete testis junction; e) tubular dilation in all regions of the testis may occur; f) spermatogenesis appears to be normal at first, but over time degenerative changes can appear; g) long term blockage of the proximal efferent ductules leads to cessation of spermatogenesis and tubular atrophy. From a practical point of view, one of the most sensitive indicators of fluid accumulation is the rapid increase in testis weight, which is often unilateral.[129-133] However, the increase in testis weight, as well as severity of the tubular dilation and degeneration depends on a number of factors, including: how many efferent ductules were occluded; time elapsed since the onset of the occlusions; dosage of the offending compound; whether the compound also has direct effects on the seminiferous epithelium; if the common duct near the epididymis is involved; and species (mice show more resistance than rats to total atrophy).[129-134]

There are several potential mechanisms that could lead to the development of efferent ductule occlusions (**Table 2**). However, microtubule disruption provides one of the best examples of this category of histopathological responses, as illustrated by a single dose of the fungicide benomyl or its metabolite carbendazim.[4,130,135] The response begins with a massive sloughing of elongated spermatids due to chemical disruption of microtubule polymerization in the Sertoli cells, which is followed by rapid transport of the sloughed cells into the epididymal lumen. Originally, it was hypothesized that the sloughed cells plugged the common efferent ductule lumen, but microdissection of treated ductules revealed that the occlusions were located primarily in the proximal region near the rete testis.[136] Furthermore, several other compounds are known to induce sloughing of germ cells without inducing occlusions;[137-141] thus, carbendazim appears to have direct effects on the ductal epithelium, as well as its known effects on the seminiferous epithelium.

The potential direct effect of carbendazim on efferent ductules appears to be through the disruption of microtubule-dependent pathways responsible for membrane recycling along the microvillus border of the nonciliated cells. Although this hypothesis has not be tested in efferent ductules, in other tissues the turnover and displacement of ion and water transport proteins was disrupted with microtubule poisons,[142-146] which could cause an increased rate of fluid resorption, sperm stasis and luminal compaction. Carbendazim has also been shown to increase the activity of Na+/K+-ATPase along the basolateral border of the nonciliated cells,[136] which could be a normal response to an increase in Na+ flux at the luminal surface. However, other potential mechanisms should also be explored. For example, a carbendazim-like sperm granuloma with seminiferous tubular

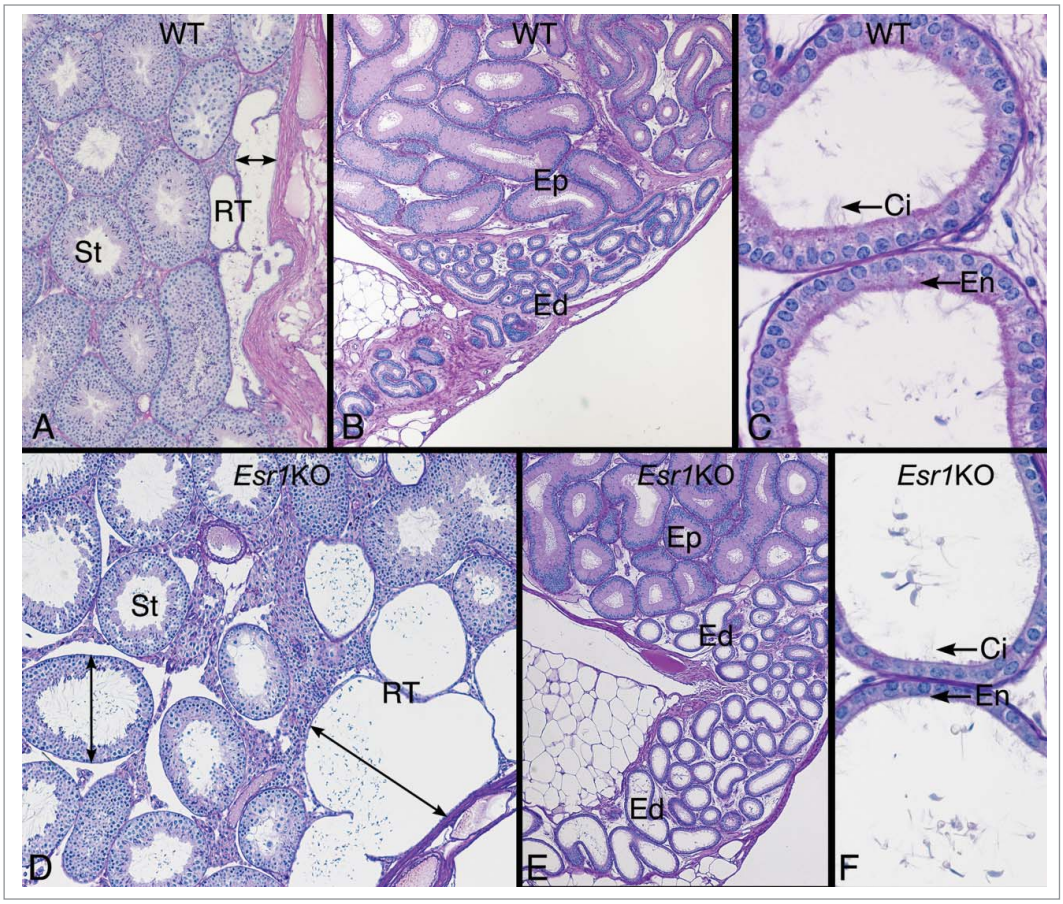

Figure 5. Testis and efferent ductules in the wild type (WT) and *Esr1*KO mice. (**A**) WT testis showing the narrow width of the rete testis and normal seminiferous tubules (St). (**B**) WT head of the epididymis region showing the coiled common efferent ductule (Ed) adjacent to the initial segment epididymis (Ep). (**C**) WT proximal region of the efferent ductules have a wider lumen than the common duct and show a PAS+ endocytic and brush border of microvilli (En) on the nonciliated cells and long cilia (Ci) protruding in the lumen from the ciliated cells. (**D**) *Esr1*KO testis showing dilated rete testis (RT) filled with fluid and causing dilation of seminiferous tubules (St). (**E**) Head of the epididymis region in the *Esr1*KO showing dilated efferent ductules (Ed) adjacent to the initial segment epididymis (Ep). (**F**) *Esr1*KO showing the dilated proximal region of the efferent ductules. The epithelium is shorter in height and appears to have lost PAS+ endocytic and brush border lining (En) on the nonciliated cells. Cilia (Ci) are noted but they appear to be thinner in density.

atrophy can be induced by a phosphodiesterase-4 inhibitor, which appears to inhibit fluid resorption rather than increase the rate of uptake.[147] The unknown factor in both cases is the stimulus for polymorphonuclear leukocyte recruitment and subsequent formation of sperm granulomas.

Multiple pathways are likely involved in the onset of granuloma formation and ductal blockage and both mechanisms could overlap in some instances. For example, it has been known for many years that α-chlorohydrin inhibits glyceraldehyde-3-phosphate dehydrogenase (G3PDH) activity in spermatozoa but also induces efferent ductule sperm granulomas, similar to those observed with carbendazim. The occlusions were thought to be due to a disruption in blood flow.[4,148] However, subsequent studies revealed that G3PDH is a microtubule-associated protein and 24 hours following α-chlorohydrin treatment β-tubulin disappears in the initial segment epithelium.[149] If a similar effect is observed in the efferent ductule epithelium, then the mechanism leading to compaction of luminal sperm and formation of sperm granulomas following α-chlorohydrin treatment may overlap with that of carbendazim and indirectly be increasing the rate of fluid resorption.

Complications of histopathological interpretations

The interpretation of histopathological changes in the testis and head of the epididymis will depend on several common factors but also differ depending on which mechanism is causing the accumulation of fluid (**Table 3**). A major complication occurs if the seminiferous tubules and rete testis are dilated, but histological sections of the efferent ductules and initial segment epididymis have not been preserved. This is a serious problem because partial or total occlusion of the efferent ductules will produce fluid accumulation in the testis similar to the *Esr1*KO mouse; however, different mechanistic interpretations are required for each condition. Another major problem is time post exposure or post development. Occlusions of the proximal efferent ductules produce rapid increases in testicular weight and dilation of the tubules.[150,151] However, when an occlusion or the inhibition of fluid resorption occurs further away from the rete testis, there can be a delay in the onset of increased testicular weight, with the delay taking up to several weeks.[6,88,89] The more distal an occlusion occurs, the greater the surface areas of normal efferent ductule epithelium that will remain for continued resorption of luminal fluid, while the ductal wall stretches in diameter to accommodate the continual release of sperm and fluid from the testis.

Prior to seminiferous tubular atrophy, testicular histopathology can show a wide range of responses to fluid accumulation following ductal occlusions, depending on numerous factors already stated. Testicular dilation may be mild to moderate, with normal spermatogenesis or severe dilation with thinning of the

seminiferous epithelium and cellular degeneration.[131,150,151] Degenerative changes in the seminiferous epithelium may included the formation of multinucleated germ cells, sloughing of immature germ cells, epithelial vacuolation, hypospermatogenesis, and apoptosis.[131,147,150-153] However, the testis and head of the epididymis have a remarkable capacity to adapt to the accumulation of fluid, as some testes having only one unobstructed efferent ductule still exhibited normal spermatogenesis in a limited number of seminiferous tubules,[134] although an increase in atrophy was noted over a 70-day period.

Species considerations are always complicated, not only from a metabolism and target organ perspective, but also because the histopathology may differ significantly, without an obvious reason. Estrogen receptor studies provide a good example. The *Esr1*KO mouse testis showed an increase in testis weight and dilation of rete testis and seminiferous tubules over an 80-day period post birth,[6] after which testis weight declined until total atrophy was observed. However, the knockout mouse was lacking ESR1 from development, therefore the pure antiestrogen ICI was used to determine if the same response would occur in the adult male. In the rat a similar time response was noted with testis weight and seminiferous tubular dilations, followed by total atrophy of the testis.[89] However, the same treatment in the pubertal mouse gave confusing results.[88] In the mouse, by day 8 post-treatment the efferent ductule lumen was dilated and epithelial structural integrity was already compromised, but the rete testis did not dilate until day 59. Furthermore, the mouse testis never increased in weight out to day 125 and atrophy was observed in only about 30% of the seminiferous tubules. Thus, the interpretation became complicated and we were never able to determine why backpressure atrophy did not occur with ICI treatment, even though the efferent ductules and rete testis exhibited nearly identical histopathological changes as seen in the *Esr1*KO mouse.

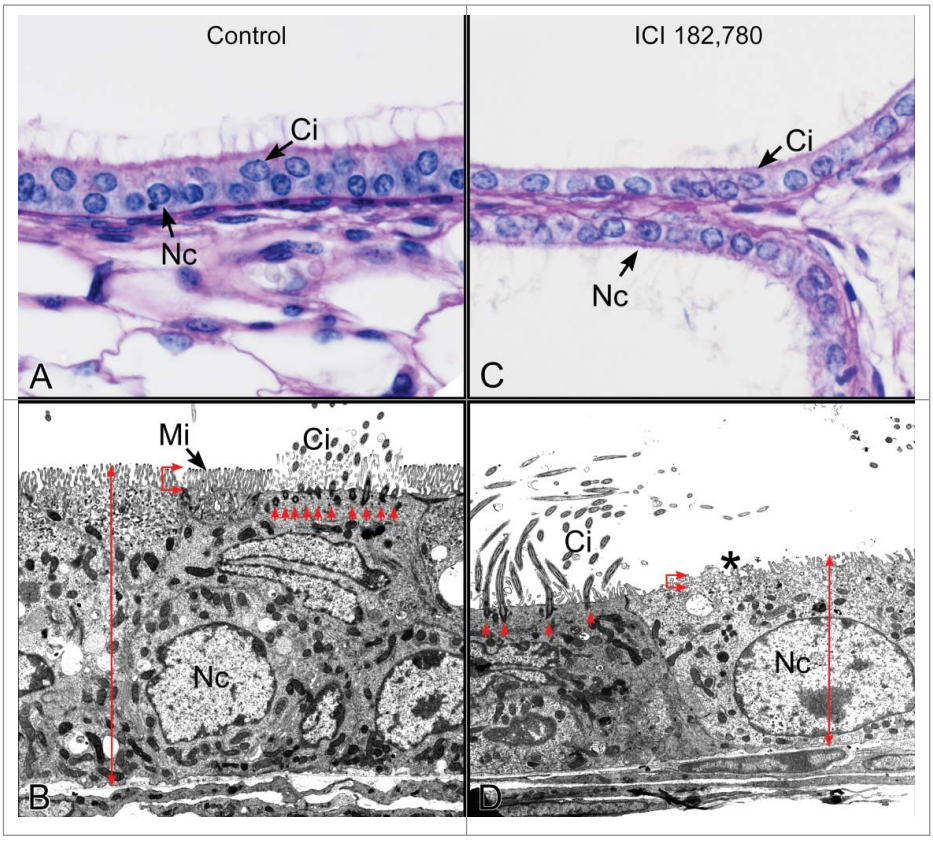

Figure 6. Efferent ductules from control and antiestrogen ICI 182,780 treated mice. (**A**) Light microscopy of the control proximal efferent ductule epithelium. Nc, nonciliated cell; Ci, ciliated cell. (**B**) Transmission electron microscopy of the control proximal efferent ductule epithelium. The nonciliated cell (Nc) has a short columnar height (double red arrow) and a prominent brush border of microvilli (Mi). The ciliated cell (Ci) has an abundance of basal bodies (red arrows) supporting the ciliary structures that protrude into the lumen. (**C**) Light microscopy of the ICI-treated proximal efferent ductule epithelium. The epithelium is shorter than normal and nonciliated cells (Nc) have a scant cytoplasm compared to the control. Ci, ciliated cell. (**D**) Transmission electron microscopy of the ICI-treated proximal efferent ductule epithelium. The nonciliated cell (Nc) is shorter in height (double red arrow) and is missing the normal finger-like projections of the microvillus border (*). The number of basal bodies (red arrows) supporting cilia (Ci) is greatly reduced.

In the case of inhibited fluid resorption, it is unclear whether tubular atrophy is due to the fluid backpressure or a direct effect of the chemical, such as the antiestrogen ICI, on the seminiferous epithelium? In the *Esr1* knock-in mouse (ENERKI), in which a point mutation in the ligand-binding domain of ESR1 allows for ligand-independent signaling,[33] the efferent ductules were basically normal but with aging the testes showed focal seminiferous tubular atrophy similar to the ICI-treated mouse. Thus, blockage or physical ligation of the proximal efferent ductules of every species will result in testicular swelling and seminiferous tubular atrophy through rapid pressure-sensitive mechanisms,[3] but long-term testicular effects of fluid accumulation following the inhibition of fluid resorption by the efferent ductule epithelium will depend on the species, the response time and other factors not yet uncovered.

Aberrant or blind-ending efferent ductules are an additional complication for histopathologists, as these small tubules are present in about 60% and 40% of the control testes/epididymides in rats and mice, respectively. The lumen of a blind-ending ductule is continuous with that of the male reproductive tract but is connected only at one end, presumably due to a failure in development from the mesonephric system of the embryo.[74,154] In rodents, blind-ending tubules are smaller in diameter, have a

Table 2. Potential mechanisms for inducing occlusions in the head of the epididymis

Cause	Potential Mechanisms[a]	References
Fluid resorption	Increase in the rate of Na+ uptake at the lumen; upregulate endothelin-1 or ET(A); increase in ESR1 expression	126, 136, 149, 166-168, 196, 228
Microtubule disruption	Indirect effect on fluid resorption; disruption of epithelial recycle of apical vesicles and membrane proteins associated with ions and water transport	136, 142, 144-146, 149, 229-235
Inflammation	Inhibition of immune tolerance; extravasation of luminal germ cells; influx of macrophages and neutrophils; stretching of ductal epithelium	2, 147, 171, 236, 237
Leakage of fluid	Damage to the tight junctions of the vascular endothelium; leakage at the efferent ductal epithelium	124, 161, 173, 174, 209
Ischemia	Inhibition of blood flow; dilation of veins; arterial occlusions; also damage to the endothelium	151, 152, 156, 173, 174, 206, 226, 227, 238
Sperm stasis	Inhibition of peritubular smooth muscle tone, either directly or indirectly through inhibition of sympathetic nerves	147, 239, 240
Developmental malformations	Abnormal growth that blocks the lumen	123, 124, 177, 178, 194, 195

[a]These are suggested mechanisms based on collective data and not necessarily direct association with efferent ductules and epididymis.

Table 3. Complications associated with histopathological interpretations of inhibited fluid resorption and sperm granulomas formation in the head of the epididymis

INHIBITION OF FLUID RESORPTION	
Potential Efferent Ductule Effects	**Histopathological Complications**
Luminal dilation	Dilation may differ depending on region of the ductule; a time-response may be involved; blind ending ducts may confuse the interpretation [3, 88, 154]
Epithelial height decrease[a]	Can be absent even with large luminal dilation [77]
Endocytic apparatus decrease[a]	Can be absent even with large luminal dilation; could miss with poor fixation [77]
Microvillus border decrease in height[a]	Can be absent even with large luminal dilation; could miss with poor fixation [77]
Potential Testicular Effects	
Testis weight increase[b]	Species and time dependent; this can be transient; correlated with tubular dilation; must examine over time; may be unilateral [6, 88, 89]
Luminal dilation of rete testis	Species and time dependent; may be induced during development; may be unilateral; could miss observation in histology section [78, 88, 89, 126]
Luminal dilation of seminiferous tubules[c]	Species and time dependent; not all tubules will show equal effects; must section rete testis region, as this region may be more severe; luminal diameter may be dilated but tubular diameter may not be enlarged; may be unilateral [88, 89, 126, 147]
Seminiferous epithelial degeneration (multinucleated giant cells, vacuolation, sloughing, hypospermatogenesis, apoptosis)	Species and time dependent; correlated with tubular dilation; must examine over time; ranges from normal to mild to severe; rete testis proximity may be more severe; may lead to atrophy [88, 89, 124, 125, 147, 156]
Atrophy of seminiferous tubules[b]	Must examine after long-term effects; not all tubules will show equal effects; may be unilateral [88, 89, 125]
INDUCTION OF SPERM GRANULOMA	
Potential Efferent Ductule Effects	**Histopathological Complications**
Luminal compaction of sperm	Dose and time dependent; not all ductules will show equal effects; proportional to dosage; may be unilateral; could miss observation in histology section [4, 130, 134, 135, 171]
Neutrophilic granulocyte inflammation	Dose and time dependent; not all ductules will show equal effects; may subside with the onset of fibrosis [4, 130, 134, 135]
Fibrosis	Must examine after long-term effects; may require serial sections
Recanalization	Must examine after long-term effects; may require serial sections [135, 241, 242]
Potential Testicular Effects	
Testis weight increase[b]	Species and time dependent; this can be transient; correlated with tubular dilation; may be unilateral; must examine over time [4, 130, 131]
Rete testis lumen dilated	Depends on location of occlusion and species; proportional to dosage; may be unilateral [129, 223-225]
Seminiferous tubular lumen dilated	Depends on location of occlusion and species; proportional to dosage; may be unilateral [129, 223-225]
Atrophy of seminiferous tubules[b]	Depends on location of occlusion and species; proportional to dosage; may be unilateral [129, 171, 223-225]

[a]Appears to be ESR1 related.
[b]Transient increase, then decrease following seminiferous epithelial degeneration.
[c]Depends on the species and age or time post treatment or developmental.

collapsed lumen with no sperm, stain more intensely but lack the typical number of lysosomes in their cytoplasm. In larger mammals, such as the dog, bull and man, the blind-ending ductules are capable of accumulating stagnant sperm, dilating in size and forming sperm granulomas.[155] Thus, the presence of these aberrant tubules must be taken into consideration when interpreting the histopathological responses observed in the head of the epididymis, but appear to be capable of contributing to ductal occlusions only in the larger species.

Conclusion

Disruption of efferent ductule epithelial function results in the accumulation of luminal fluids that is capable of backpressure into the rete testis and seminiferous tubules, causing transient dilation, epithelial degeneration and even testicular atrophy. This histopathological sequence was originally discovered following surgical ligation of the efferent ductules or treatment with chemicals that induced sperm granulomas in the head of the epididymis. However, a similar morphological sequela in the testis was also observed following the disruption of ESR1 function in the efferent ductules, which revealed the importance of preserving these delicate ducts for evaluation, but also brought attention to the role that estrogen plays in maintaining fluid resorption by the efferent ductal epithelium. Although efferent ductules are difficult to preserve for routine histological sectioning,[2] their evaluation is essential for determining the mechanism of testicular injury if dilation is observed in the rete testis and/or seminiferous tubules, but also when unexplained seminiferous tubular atrophy is present in a long term study. Backpressure atrophy of the testis can be rapid and once the efferent ductules are occluded the lesion appears to be permanent.[4]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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