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Estrogens and development of the rete testis, efferent ductules, epididymis and vas deferens

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Abstract

Estrogen has always been considered the female hormone and testosterone the male hormone. However, estrogen's presence in the testis and deleterious effects of estrogen treatment during development have been known for nearly 90 years, long before estrogen receptors (ESRs) were discovered. Eventually it was learned that testes actually synthesize high levels of estradiol (E2) and sequester high concentrations in the reproductive tract lumen, which seems contradictory to the overwhelming number of studies showing reproductive pathology following exogenous estrogen exposures. For too long, the developmental pathology of estrogen has dominated our thinking, even resulting in the "estrogen hypothesis" as related to the testicular dysgenesis syndrome. However, these early studies and the development of an *Esr1* knockout mouse led to a deluge of research into estrogen's potential role in and disruption of development and function of the male reproductive system. What is new is that estrogen action in the male cannot be divorced from that of androgen. This paper presents what is known about components of the estrogen pathway, including its synthesis and target receptors, and the need to achieve a balance between androgen- and estrogen-action in male reproductive tract differentiation and adult functions. The review focuses on what is known regarding development of the male reproductive tract, from the rete testis to the vas deferens, and examines the expression of estrogen receptors and presence of

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aromatase in the male reproductive system, traces the evidence provided by estrogen-associated knockout and transgenic animal models and discusses the effects of fetal and postnatal exposures to estrogens. Hopefully, there will be enough here to stimulate discussions and new investigations of the androgen:estrogen balance that seems to be essential for development of the male reproductive tract.

Keywords

Estrogen; estrogen receptor; development; differentiation; fetal; neonatal; male reproduction; testis; rete testis; efferent ductule; epididymis; vas deferens; mesonephros; mesonephric tubule; Wolffian duct; rete cord; environmental estrogens; testicular dysgenesis syndrome

1. Introduction

We are currently at something of a crossroads in terms of our understanding of the importance of estrogens in the development and subsequent function of the male reproductive tract. To appreciate how we have reached this point, it is helpful to briefly review the history of the area. For many years, the scientific community assumed that estrogen was the female sex steroid, while testosterone (T) was the male sex steroid and, in females, was only used as an intermediate in the synthesis of estradiol (Eik-Nes, 1964). However, it has been known since 1921 that the testis produces a substance capable of increasing uterine weight and thus having a feminizing action (Fellner, 1921). Indeed, the first isolation of estradiol (E2) in the male was from the horse testis (Beall, 1940). Subsequently, numerous studies found high concentrations of estrogens, particularly estrone-sulfate, in the testis, rete testis fluid, semen and blood of the horse and other large domestic species (Amann and Ganjam, 1976; Claus et al., 1992; Claus and Hoffmann, 1980; Claus et al., 1985; Eiler and Graves, 1977; Setchell and Cox, 1982; Setchell et al., 1983). Even in the rodent, the concentration of E2 in rete testis fluid was reported to reach 510 pg/ml (Free and Jaffe, 1979), which is 5-fold higher than serum E2 in the female rat during proestrus (Döhler and Wuttke, 1975; Overpeck et al., 1978).

Long before the isolation and cloning of estrogen receptors (ESR), the actual presence of a ‘female’ hormone in the male raised curiosity about its source and potential function, but most research was focused on the effects of estrogen administration in males during development. Before the 1990s, most studies of estrogens in the male were devoted to describing reproductive pathologies induced by neonatal exposures to various types of estrogens, as well as to the synthetic, non-steroidal, but potent estrogen, diethylstilbestrol (DES) (Arai et al., 1978; Bern, 1951; Brown-Grant et al., 1975; Bullock et al., 1988; Dhar and Setty, 1976; Dunn and Green, 1963; Gill et al., 1977; Hendricks and Gerall, 1970; McLachlan et al., 1975; Newbold et al., 1985, 1987; Ohta and Takasugi, 1974; Takasugi, 1970; Yasuda et al., 1985; Zadina et al., 1979). The seeming contradiction between the many reports of physiologically high E2 production by the testis/reproductive tract on the one hand and, on the other hand, the overwhelming evidence of male reproductive pathology following exogenous estrogen exposures during development, might have been expected to cause researchers to pause and reflect. Instead, thinking was effectively steamrollered when

the so-called ‘Oestrogen hypothesis’ was published in 1993 (Sharpe and Skakkebaek, 1993). This paper, which hypothesized that inappropriate estrogen exposure during perinatal development might underlie common human male reproductive pathologies (e.g., low/falling sperm count, undescended testis and hypospadias), established a new paradigm in developmental biology. The coincidence of this hypothesis with emerging studies showing that a variety of common environmental contaminant/pollutant chemicals possessed intrinsic (though weak) estrogenicity, was pivotal in kick-starting the era of endocrine-disrupting chemicals, which is still with us.

Whilst these developments served to hugely increase interest in estrogen’s adverse effects, they rather sidelined thinking about the physiological importance of estrogens in males. This changed with the discovery that knockout of the one known estrogen receptor (ESR1) at the time, resulted in dramatic changes in the male reproductive tract and infertility (Eddy et al., 1996; Hess et al., 1997a; Lubahn et al., 1993). Such developments led to an explosion in research that has provided an array of discoveries, including the following: a) an understanding of where and when E2 is produced in the male, and better antibodies for the localization of ESR (see previous reviews: Cooke et al., 2017; Hess and Cooke, 2018); b) discovery of a second receptor-ESR2 (Kuiper et al., 1996); c) the generation of several key knockout mice, which demonstrated an absolute requirement for the expression of ESR1 in male reproduction; and d) an abundance of environmental studies showing detrimental effects of synthetic chemicals on male reproductive tract development (Basak et al., 2020; Belcher et al., 2019; Conley et al., 2018; Lymperi and Giwercman, 2018).

Despite these discoveries, it is fair to say that the predominant research view at the start of this millennium was that developmental exposure of the male to estrogens in any form is likely to have negative consequences, largely ignoring the evidence to the contrary from *Esr1* knockout (KO) mice. It is emphasized that this (imbalanced) perspective stemmed from research that had operated at the ‘extremes’, on the one hand exposing developing rodents to pharmacologically high doses of exogenous estrogens that induced adverse effects, while on the other reducing endogenous estrogen action (via *Esr1*KO) to zero, but also inducing adverse effects. The physiological importance of estrogens in the male is somehow lost in the middle of this ‘research at the extremes’. A further complication that then emerged was the discovery that the adverse developmental effects of high-dose DES on the male reproductive tract resulted from functional wipe-out of androgen action due to a double whammy effect of grossly reducing testicular production of T, at the same time it was wiping out expression of the androgen receptor (AR) at the protein level. This was proven by co-administration of T with the high dose DES, which essentially prevented all the adverse effects of DES (McKinnell et al 2001; Rivas et al 2003). These same authors went on to show that the adverse effects of DES resulted from disruption of the androgen-estrogen balance (Rivas et al 2002), which is discussed in more detail later.

What these new studies did was to highlight that estrogen action in males cannot be divorced from androgen action. Indeed, substantial research has gone on to identify that the pivotal event for normal reproductive development of the male is to be exposed to a sufficient level of androgens during a specific fetal period, the ‘masculinization programming window (MPW)’ (Sharpe, 2020). There is no current evidence that estrogens are involved in this

process, but as this paper will show, all of the components for estrogen production and action are present in the developing male reproductive tract at this stage of development, so a physiological role for estradiol, or for the balance between androgen- and estrogen-action or their respective receptors is likely.

So, now we have come full circle and must ask again, “how important are estrogens in the development of the male reproductive tract and what are its specific physiological roles and contribution to male fertility?” As this review will show, we still have many more unanswered than answered questions in this regard. The review focuses on what is known regarding development of the male reproductive tract, from the rete testis to the vas deferens, examines the expression of estrogen receptors and the production of estrogen in the male reproductive system, traces the evidence provided by estrogen-associated knockout and transgenic animal models and discusses the effects of fetal and postnatal exposures to estrogens. Finally, we hope to stimulate the next generation of scientists by discussing where and when estrogen and/or an estrogen/androgen balance might play a role in the development of the male reproductive tract.

2. Morphogenesis of the embryonic mesonephric tubules/efferent ducts and mesonephric/Wolffian duct and their connections

Overview

Morphogenesis of the rete testis cord, its connection to mesonephric tubules, which will become the efferent ducts, and development of the Wolffian duct (mesonephric/nephric duct), which will become the epididymis and vas deferens, is complex and involves early expression of steroid receptors and synthesis of T and E2 (Fig. 1). The process involves a series of events that result in the efferent ducts having a unique branching pattern, which is quite different to the morphogenesis of classic branching tissues such as the prostate and mammary gland. On the other hand, the morphogenesis of the Wolffian duct is via non-branching morphogenesis, and the mechanisms by which this occurs may resemble that of the brain and intestine. It is unclear as to whether tissues that form biological tubules and then undergo their morphogenic events to form adult structures utilize unique mechanisms or there are overlapping mechanisms between the tissues (see further by: Lubarsky and Krasnow, 2003). It is possible both are used in that certain overall events are common, for example, cell proliferation and cell rearrangements but these events are differentially regulated. Although beyond the scope of this review for further discussion, it is also unclear as to the mechanisms that dictate the size and shape of these tissues. How does the Wolffian duct form the “dumbbell/hour glass-shape” seen in the adult epididymis of several species, and what limits its size?

Following the development of the three germ layers, endoderm, mesoderm and ectoderm, the mesoderm (Greek: *meso*-middle, *derma*-skin) divides into three regions, paraxial, intermediate and lateral plate, of which the intermediate is the most important for this discussion. A series of events from anterior to posterior in sequence will form the pronephros, mesonephros and the metanephros, and it is the mesonephros that will go on to form the mesonephric tubules and duct.

However, the development of the mesonephric tubules, for example, is not “intermediate mesoderm/mesonephros autonomous” in that mid-line structures such as notochord and floor plate interact with the paraxial mesoderm via signaling pathways, which in turn again, via signaling pathways (e.g. Sonic hedgehog; *Shh*), regulate tubulogenesis and numbers of mesonephric tubules (Murashima et al., 2014).

Development of the mesonephric duct and mesonephric tubules

Within the most anterior region of the mesonephros a group of cells undergo specification through an unknown mechanism, take on a migratory phenotype and migrate towards the cloaca eventually forming the mesonephric/Wolffian duct. The migratory cells are under the control of a Fibroblast Growth Factor (FGF) gradient, at least in the chick (Atsuta and Takahashi, 2015; Attia et al., 2015) with the highest concentration of FGF being found at the cloacal end. As the animal grows, the cells within the proximal region find themselves surrounded by a lower concentration of FGF, which triggers signal transduction pathways leading to mesenchymal-epithelial transition, epithelial cell polarization and the eventual formation of a tube/duct. This continues until the entire group of migratory cells have undergone these processes, thereby forming a definitive duct. It is tempting to speculate that a similar mechanism is found during the formation of the human mesonephric duct. In the mouse, fusion of the Wolffian duct with the cloaca has been shown to be via a mutual apoptotic event (Hoshi et al., 2018). As the migratory Wolffian duct cells approach the cloaca, some of those cells together with a subset of cells within the cloaca undergo apoptosis, which allows the two structures to unite (See for a review on the mechanisms by which biological tubes fuse/join: Kao, 2013).

During migration of the Wolffian duct towards the cloaca, it produces Wnt9b (Carroll et al., 2005), which acts upon a group of cells within the surrounding mesenchyme to undergo mesenchymal-epithelial transition to form simple vesicles and then, S-shaped vesicles/bodies. Loss of *Wnt9b* results in loss of induction of the mesenchyme to form mesonephric tubules (Carroll et al., 2005). These vesicles are very similar to the vesicles that form renal nephrons. Renal vesicles that form within the metanephric mesenchyme undergo extensive patterning to generate S-shaped bodies having four regions: a region that connects to the Wolffian duct, distal, medial and proximal regions with each having overlapping and unique gene expression patterns along the length of the body (Georgas et al., 2009; Rumballe et al., 2011). Such detailed studies have not been performed examining the patterning of the S-shaped bodies that will eventually form the efferent ducts, but some specific markers have been identified in the proximal and distal regions of the S-shaped body (Sainio, 2003; Sainio et al., 1997). Studies have also shown some similar and unique overall gene expression profiles between the S-shaped bodies that will form metanephric renal nephrons and the efferent ducts. As mentioned above, *Shh* plays an indirect role in regulating the number of mesonephric tubules within the intermediate mesoderm. In addition to *Wnt9b* and *Shh*, *Six1*, *Wt-1*, *Foxc1*, *Foxc2* and *Robo2* are also important in regulating the morphogenesis of the mesonephric tubules (for extensive review see: Murashima et al., 2015). Most of the studies focusing on the development of human mesonephric tubules have been from a structural perspective. For example, human S-shaped bodies have been examined by late 19th century to early 20th century embryologists (Lewis, 1920; Meyer, 1890) and more recently by

Ludwig & Landmann (2005). Lewis (1920) showed three-dimensional models of human mesonephric tubule development, with S-shaped bodies, being referred to as “double spirals,” connected to a glomerulus at one end and to the Wolffian duct at the other end. In addition to the models, Lewis (1920) suggested that the most proximal part of the tubule was secretory in function, whereas the most distal (closest to the testis) was a collecting part. It was shown that the secretory part of the tubule underwent regression, the collecting part then underwent elongation eventually joining the rete testis (Jacob et al., 2012). Some genes have been identified that are expressed in human mesonephric tubules and include *Jagged1* (Crosnier et al., 2000), *anosmin1* (Hardelin et al., 1999), EGF, TGF-alpha and receptors (Bernardini et al., 1996), although their role(s) are unknown. Interestingly, Lawrence and colleagues (Lawrence et al., 2018) have shown influx and efflux of organic anions and cations across mouse mesonephric tubule epithelium. This movement of anions and cations could reflect an excretory function, although further studies are needed to examine this possibility.

Mesonephric tubules destined to become the efferent ducts

As development proceeds, the caudal (posterior) mesonephric tubules undergo regression leaving 4–6 tubules in close apposition to the testis, which will form the efferent ducts. The manner by which those tubules survive is not entirely clear but some studies have suggested that the cells produce anti-apoptotic proteins whereas caudal tubule cells that undergo regression do not, but produce pro-apoptotic proteins (Sainio et al., 1997). Another study has shown that the caudal mesonephric tubules undergo regression via activation of cell senescence pathways, but the cranial tubules do not express the components of that pathway and are so, “protected” from regression (Muñoz-EspÌn et al., 2013). Presumably, the mechanisms by which the mesonephric tubules survive are dependent upon some factor(s) secreted by the testis, which in turn initiate survival pathways. Interestingly, *Wt1* is important for the survival of the caudal tubules but not the cranial tubules, thereby showing differential regulation between the two sets of tubules (Kreidberg et al., 1993; Sainio et al., 1997).

In the mouse, the induced mesonephric tubules join the Wolffian duct between E9.5 and E11.5 (Vetter and Gibley, 1966), and then join the rete testis by E15.5 (Karl and Capel, 1995; Omotehara et al., 2020). Mesonephric tubules form lumens early in development, with small lumens at E11.5 in mice (Omotehara et al., 2020) and in humans at 9 weeks (Cunha et al., 2020). The mechanism(s) by which the tubules join with the rete testis and Wolffian duct are unclear and could certainly be similar to the manner by which the Wolffian duct joins the cloaca. Clues as to the mechanism(s) by which the mesonephric tubules join the rete testis have come from recent studies of several laboratories (Combes et al., 2009; Kulibin and Malolina, 2020; Omotehara et al., 2020). Combes et al. generated three-dimensional models of the testis and mesonephric tubules from E15.5 mouse embryo and showed the connection between the two tissues (Fig. 2). A plexus containing perforations was identified at the connecting points. Later, Omotehara et al (2020) showed very nicely the connection between mouse adrenal-4 binding protein/steroidogenic factor-1 positive gonadal somatic cells within the rete cords and the cells within the mesonephric tubules. Once contact was made between

the two cell types, a common basement membrane containing collagen type IV surrounded the mesonephric tubules and the rete.

The following question arose while writing this review: when do the mesonephric tubules become efferent ductules? It could be assumed that they remain mesonephric tubules while *in utero*, but that would suggest some magical differentiation taking place at birth, when in fact even the ciliated efferent ductal cells do not differentiate until PND5 in the mouse (Benoit, 1926). However, the general function of efferent ductules is to connect rete testis to the epididymis and as such this function occurs during fetal development and in mice at E15.5 (Karl and Capel, 1995; Omotehara et al., 2020). Therefore, we propose that this event be used for initial labeling of the tubules as efferent ductules.

From E15.5 onwards, the mesonephric tubules/efferent ductules elongate and coil, and unite/fuse/join with each other forming their characteristic branching pattern (can be reviewed in: Guttroff et al., 1992; Ilio and Hess, 1994). As mentioned in the Introduction, the morphogenesis of the branching pattern appears to be unusual in that it is not similar to that observed in tissues that undergo classical branching morphogenesis. We hypothesize that as the mesonephric tubules elongate and coil, they fuse with each other in a relatively non-stochastic pattern, that is, there is always a single communicating duct with the epididymis and at least 4–6 tubules (for rodents, for example) attached to the rete. However, it is partly stochastic in that the branching pattern can vary between males of the same species and also, between species. An excellent illustration of this point is from the work of Nakata & Iseki (2019), that showed in two mice of the same strain having the characteristic 4 tubules attached to the rete, which in turn attached to form pairs, which in turn attached to form a single pair, which in turn attached to form a single communicating duct/tubule. Blind ended tubules were not observed in these two samples. However, in another mouse, the pattern was similar but there were numerous blind-ending tubules. Blind-ending tubules were found in 60% and 40% of male rats and mice, respectively (Guttroff et al., 1992; Hess et al., 2000). In rodents, these small tubules exhibited abnormal epithelial morphology and had very small lumens that did not accumulate sperm, in contrast to larger mammals (Hess, 2002), which tend to form expanded sperm granulomas and cysts, as seen in the human (Mennemeyer and Mason, 1979). It is not clear how blind ended tubules form but presumably it is the failure of those tubules to fuse/join to a nearby tubule. Because the mesonephric tubules and differentiated efferent ductules express an abundance of ESR1 (section 3), it is possible that the estrogen receptor signaling pathway is involved in growth and branching of the mesonephric tubules. This hypothesis was supported by an unexpected observation in the *Esr1*KO mouse, which revealed 100% incidence of blind ended tubules compared to 40% in wild-type mice and the abnormal tubules were longer and contained bulbous endings in the *Esr1*KO males (Hess et al., 2000).

The mechanisms by which the mesonephric tubules elongate and coil are also not known but it is tempting to speculate that cell proliferation coupled with cell rearrangements as observed in the Wolffian duct (see below) are responsible. Interestingly, closer examination of the three-dimensional models of the adult mouse efferent ducts suggest that each tubule coils in isolation, they do not appear to coil around each other (Lambot et al., 2009; Nakata and Iseki, 2019). One clear distinction between human efferent ducts and the efferent ducts

from other animals is that the efferent ducts in the human form the majority of the head (caput) region of the epididymis (Sullivan et al., 2019; Yeung et al., 1991). In rodent species, only the single common duct penetrates the epididymal capsule and coils near the initial segment (Hess, 2002, 2018a). This is the reason why the head of the human epididymis is of mesonephric tubule origin and not of mesonephric duct origin.

Update on the regulation of Wolffian duct elongation and coiling

For a more extensive discussion on the development of the Wolffian duct, the reader should refer to several reviews (de Mello Santos and Hinton, 2019; Georgas et al., 2015; Hannema and Hughes, 2006; Hannema et al., 2006; Hinton and Avellar, 2018; Hinton et al., 2011; Joseph et al., 2009; Murashima et al., 2014; Shaw and Renfree, 2014). At E14.5 in the mouse, the Wolffian duct is a single unconvoluted tubule approximately 1 mm in length, and will elongate to approximately 1 meter in the adult. The length of the human epididymal duct is 6 meters (Hinton et al., 2011; Sullivan et al., 2019). Coiling is observed at E15.5 beginning in the proximal region of the tubule in mice (Hinton and Avellar, 2018), which is mostly a simple sine-like wave. Very nice studies by (Hirashima, 2014, 2016) suggests that the biomechanical properties of the duct, the surrounding mesenchyme and capsule contribute to coiling. It is beyond the scope of this review to discuss this further, but this is an exciting finding since biomechanical properties play key roles during elongation and coiling of other tissues, for example the brain and heart. However, it is important to recognize that cell proliferation and cell rearrangements will contribute to the biomechanical properties of the elongating and coiling epididymal duct.

Certainly, cell proliferation is a major contributor to epididymal ductal elongation, and more recently, it was recognized that cell rearrangements, for example, convergent extension type of movement also contributes to duct elongation (Xu et al., 2016b). In the latter case, Protein Tyrosine Kinase 7, which is a member of the non-canonical Wnt pathway, is a major regulator (Xu et al., 2016a). Other members of the Wnt signaling pathway have also been shown to be major regulators of Wolffian duct elongation and coiling (Kumar et al., 2016) as have *Fgf8* (Kitagaki et al., 2011), *SPAG11C* (Ribeiro et al., 2017), *Hox* genes (Branford et al., 2000; Raines et al., 2013; Zhao and Potter, 2002) and *Inhba*. (Tomaszewski et al., 2007). The potential involvement of estrogens in coiling and elongation of the Wolffian duct is suggested by studies showing that neonatal over-exposure to estrogens can reduce elongation/coiling within the epididymis and blurring of the epididymal-vas deferens transition as evidenced by coiling of the proximal vas, changes that are associated with altered epithelial and stromal expression of ESR1 (Atanassova et al., 2001; Atanassova et al., 2005b).

Perhaps the most well-recognized regulator of Wolffian duct development is testosterone and Fig. 1 shows key events of the action of androgens. The figure also shows the key events of the action of estrogen and despite the well-known action of this hormone on the postnatal development of the efferent ductules and epididymis (Hess and Cooke, 2018), it is unclear as to whether estrogen plays a significant role during the embryonic period (see sections 3–4). Data from the *Esr1*KO mouse strongly suggest that estrogen, or at least the expression of ESR1, has an important role in the differentiation of epithelia, in addition to potential effects

on branching and the number of blind ended tubules formed. In wild-type males, the efferent duct epithelium terminates abruptly where the initial epididymal segment begins, but in the *Esr1*KO mice blotchy portions of initial segment epithelium were observed in the common efferent duct, as well as near the rete testis (Hess et al., 2000; Joseph et al., 2011), which suggests epithelial differentiation and the expression of region-specific genes may be under estrogen, as well as androgen, regulation.

Estrogen regulates several genes in breast cancer cells/tissue that are also expressed in the developing male reproductive tract, for example, *Pax2* (Beauchemin et al., 2011) and *Gata3* (Wilson and Giguère, 2008). Several genes appear to be highly expressed in mouse mesonephric tubules (Snyder et al., 2010) from E14.5 to P1 that are possibly regulated by estrogen, including the following: gene regulated by estrogen in breast cancer protein (*Greb1*), sulfotransferase 1E1 (*Sult1e1* and *Sult1c2*), DEAD box 5 (*Ddx5)* an RNA helicase (Xing et al., 2019)) and UDP glucuronosyltransferase 1 family, polypeptide A1 (*Ugt1a1*). It is anticipated that a Wolffian duct and mesonephric tubule phenotype will be observed in *Greb1* knockouts, because Greb1-like (*Greb1l*) knockouts (De Tomasi et al., 2017) clearly show a loss of the Wolffian duct and the most anterior mesonephric tubules that will form the efferent ductules. Expanding this thought further, it is tempting to speculate that *Greb1* also plays a role in cell rearrangements during Wolffian duct and mesonephric tubule elongation because this gene is important during axial elongation in zebrafish embryos (Prajapati et al., 2019).

3. Estrogen receptors in the Male reproductive system

Estrogen receptor presence in the adult male reproductive tract has been thoroughly reviewed and the reader is urged to examine the following papers: (Carreau and Hess, 2010; Cooke et al., 2017; Hess, 2004; Hess and Carnes, 2004; Hess and Cooke, 2018; Hess et al., 2011; O'Donnell et al., 2013). Here we will focus on the fetal and neonatal presence of ESR1 and 2, as well as androgen receptor (AR) in the human, rat and mouse. However, for comparisons adult ages are included. Also, this section will include the testis, because the rete testis in man is mediastinal and penetrates approximately1/3 of the testicular core (Hess and Hermo, 2018).

The presence of high concentrations of E2 in the testis and rete testis fluid (Table 1) and the observation of pathological changes in the male reproductive system, following perinatal estrogen treatment (Brown-Grant et al., 1975; Dunn and Green, 1963; Emmens and Parkes, 1947; Greene et al., 1938; Hendricks and Gerall, 1970; Kincl et al., 1963; Kincl and Maqueo, 1972; McLachlan et al., 1975; Mori, 1967; Takasugi, 1970), suggested to many that estrogen receptors must be present in the male reproductive system, even during development (Iguchi, 1991). Evidence for ESR presence began to appear first in ^{3}H-E2 binding studies that demonstrated distinct high affinity in interstitial cells of the testis and in whole epididymal extracts (Brinkmann et al., 1972; Danzo et al., 1975; Danzo et al., 1978; Danzo et al., 1977; Mulder et al., 1973; Mulder et al., 1974a; Mulder et al., 1974b; Schleicher et al., 1984; Stumpf, 1969; Stumpf and Sar, 1976; Toney and Danzo, 1988). At the time, it was assumed that only one ESR existed (Nilsson et al., 2001), but this original data, although encouraging for the general hypothesis, became less interpretable when a

second ESR (ERβ) was cloned from the male rat prostate (Kuiper et al., 1996), as E2 binding could easily target ESR1 or ESR2 (see review: (Nilsson et al., 2001)). However, as it turns out, loss of ESR2 in the knockout mouse showed no major effects on the development of testes, efferent ducts and epididymis, although prominent long-term effects were seen in the prostate and an increase in neonatal gonocytes, but minor effects in the adult testis (Chen et al., 2010; Delbes et al., 2006; Delbes et al., 2004; Gustafsson et al., 2019; Krege et al., 1998; Prins and Korach, 2008). Therefore, the major focus here will be the presence and function of ESR1 in the male, during the fetal and neonatal periods.

Determining specific expressions and localization of ESR1 and ESR2 in the male has been difficult and remains challenging today. Wide variation in reported presence or absence for steroid receptors in the male tract, including that of androgen receptor (AR), are seen in Tables 2–4. Differences in specificity of antibodies, as well as changes in results depending on tissue fixation and processing, including antigen retrieval methods (Andersson et al., 2017; Cooke et al., 2017; Gustafsson et al., 2019; Iwamura et al., 1994; Nelson et al., 2017; Shi et al., 1993), have led to these disparities. Thus, drawing conclusions and interpreting data from estrogen treatment-related effects, in specific male reproductive tissues, has become problematic. Some labs have reported no ESR1 mRNA in fetal and neonatal human testes (Berensztein et al., 2006; Gaskell et al., 2003), but an abundance of mRNA and protein were found in the mouse (Cederroth et al., 2007; Jefferson et al., 2000; Mowa and Iwanaga, 2001; Nielsen et al., 2000). Some laboratories find ESR1 expressed in neonatal rat Sertoli cells (Lucas et al., 2008), while others report none (Fisher et al., 1997; Sar and Welsch, 2000). Probably most troubling are conflicting reports for ESR mRNAs. However, despite this struggle, some patterns of receptor localization can be inferred. For example, it has been repeatedly confirmed in every species examined to date that efferent ductules express the highest concentration of ESR1 of any tissue (Cooke et al., 2017; Hess and Cooke, 2018), male or female, and does so starting during early fetal development of the mesonephric tubules (Fig. 3).

Binding of E2 in male fetal reproductive tissues occurs prior to or simultaneous with androgen binding and occurs first in the efferent ductules (Cooke et al., 1991b). Subsequent immunohistochemical staining has shown that ESR1 appears more prominent in the efferent ductule epithelium, while ESR2 can be more prominent in the testis and epididymis (see Table 2 and reviewed in: Cooke et al., 2017), although specific cell types show more intense staining for ESR1 (Zhou et al., 2002). ESR1 expression is quite strong in the mesonephric/efferent ductule epithelium from the fetal and neonatal period through to adulthood, but the stromal areas are either negative or weakly positive (Fig. 3). In contrast, the rete testis appears to be either negative or weakly positive until adulthood, when the epithelium can show strongly positive staining. The Wolffian duct/epididymal epithelium is also negative to weakly positive for ESR1 during fetal and neonatal development, but then shows increased staining near puberty in some reports. A consistent observation for the epididymal epithelium is an intense expression of ESR1 in the apical and clear cells. For both the efferent ducts (in some species) and epididymis, it appears that the stromal areas can be more intensely stained during fetal development. The vas deferens epithelium has also been reported as negative for ESR1, while the stroma has shown some positivity during development.

The classical concept of estrogen-mediated signaling involves a ligand-dependent transcription factor or receptor that dimerizes, becomes phosphorylated and translocates to the nucleus, where the activated ESR binds to estrogen response elements in the DNA of specific genes. The modulation of transcription is achieved through its activation function domains (AF-1 and AF-2) that recruit coregulators in a complex interaction and docking on specific regions of the DNA (Arnal et al., 2017; Korach et al., 2019). This classical estrogen pathway requires both the production of E2 via the enzyme aromatase and the expression of nuclear ESRs. Therefore, great effort has been made to understand the developmental expressions of aromatase, as well as the receptors, ESR1 and 2 (Tables 2–4). However, this original pathway was found to be incomplete, as there have been new discoveries of non-genomic estrogen activity, including extranuclear or membrane ESR paths, as well as ligand-independent signaling, often involving growth factor activation of ESRs (Arnal et al., 2017; Levin, 2015; Stefkovich et al., 2017; Stellato et al., 2016).

One of the more surprising discoveries was that loss of the ability to produce E2, the natural ligand of ESR1, in the aromatase (*Cyp19*) KO (AromKO) mouse, had no effect on male reproductive tract development and physiology and produced relatively normal fertility until the mice began to age (Robertson et al., 1999; Robertson et al., 2002; Robertson et al., 2001). However, the expression of just the receptor, ESR1, was found to be essential for development and function of the male reproductive tract (Eddy et al., 1996; Hess et al., 1997a; Hess et al., 2000; Joseph et al., 2010a; Joseph et al., 2010b; Lee et al., 2000; Lee et al., 2009; Lubahn et al., 1993; Nakai et al., 2001; Toda et al., 2008; Zhou et al., 2001) and its expression was not altered in the AromKO male (Toda et al., 2008; Toda et al., 2001). This serendipitous discovery that the unliganded ESR1 can maintain efferent ductule structure and function demonstrated the regulation of essential genes in the absence of the steroid ligand (Caizzi et al., 2014; Sinkevicius et al., 2009; Stellato et al., 2016). Very little is known about estrogen regulation of early development of the testis and reproductive tract; however, during *in utero* development, ESR1 expression in the mesonephros appears to occur prior to the expression of aromatase in the testis. Thus, an unliganded ESR1 may have a role in the early differentiation and migration of mesonephric tubule epithelial cells. This conclusion is supported by an *in vitro* study demonstrating that ESR1 can bind to more than 4,000 chromatin sites in the absence of E2 (Caizzi et al., 2014). Importantly, these binding sites were shown to be related to genes linked to development and differentiation (Stellato et al., 2016).

In light of these stated complications and surprising discoveries related to ESR1, we are left with numerous questions, including the following. What is the precise timing of ESR1 and AR expression during fetal development and how do these compare across species? How do we successfully analyze ESR antibody data and determine which techniques are best for preservation of cellular localization? Will single-cell RNA sequencing and single cell proteomics finally resolve the conflicting data of ESR1 presence in the male reproductive tract (Green et al., 2018; Tan et al., 2020)? When will experiments finally determine the biochemical and physiological nature of co-localization of AR and ESR1 in the same cell? Also, why are the membrane and nuclear ESR1s equally essential for development of the efferent ductules and head of the epididymis (Cooke et al., 2019; Nanjappa et al., 2016)? Finally, how does the unliganded ESR1 actually function in efferent ductules? Is it possible

that the expression of AR alongside an unliganded ESR1 in the presence of T provides cooperativity in the maintenance of specific genes? For example, both estrogen and androgen response elements are found in the promoter region of genes responsible for epithelial maintenance of fluid reabsorption in efferent ductules (Hess and Cooke, 2018; Snyder et al., 2009; Trepos-Pouplard et al., 2010; Yao et al., 2017). Many questions remain unanswered, but one intriguing fact stands out: ESR1 is essential and constitutively expressed in parts of the male reproductive tract (Oliveira et al., 2004), but the male reproductive system develops in the absence of its natural ligand E2 (Toda et al., 2008).

4. Aromatase and synthesis of estrogen in the male reproductive system

Aromatase cytochrome P450, CYP19A1, which catalyzes the final step of androgen conversion to 17β-estradiol (E2) and estrone (E1) (Corbin et al., 1988), is present in the testis from fetal development through to adulthood (Borday et al., 2013; Brodie and Inkster, 1993; Carreau and Hess, 2010; Dorrington and Armstrong, 1975; Guercio et al., 2020; Nitta et al., 1993; O'Donnell et al., 2001; Payne et al., 1987; Weniger and Zeis, 1988). During fetal development, serum E2 concentrations (Table 1) are relatively high in human males and females, as well as rodent species (Bonagura et al., 2011; Habert and Picon, 1984; Robinson and Bridson, 1978; vom Saal et al., 1997). In the male, this elevated level of estrogen continues neonatally (Table 1), when E2 is much higher than in the adult (Döhler and Wuttke, 1975; Gorski et al., 1977; Overpeck et al., 1978; Rommerts et al., 1982). In one rat study, E2 was reported to be over 300 pg/ml on postnatal day (PND) 1, but declined to near 50 pg/ml on day 3, followed by another rise just before weaning, and finally a steady decline toward ~25 pg/ml in the adult (Döhler and Wuttke, 1975), approximately the same concentration reported in adult men (Finkelstein et al., 2013). Thus, the fetal and neonatal periods of development in the male experience a significantly elevated level of estrogen, but also rising levels of T (Habert and Picon, 1984), which raises numerous questions regarding the sources and functions of E2 in organs that depend so much on androgens for activation and maintenance of functions programmed in fetal life during the 'masculinization programming window' (see review: Sharpe, 2020). Another question that needs attention, but cannot be addressed here, is the high levels of serum alpha-fetoprotein that binds E2 with high affinity in rodents and could limit the influence of circulating estrogen over target organs in the male (Cunha et al., 2019). However, this also places a greater emphasis on aromatase's expression locally, such as the testis, which could diffuse throughout the developing gonad and mesonephros. Finally, the topic of sulfated or conjugated estrogens and estrogen- (SULT1E1) and hydroxysteroid- (SULT2A1) sulfotransferases also requires greater attention than has been given in the past. Sulfoconjugation can block ESR1 binding and thus the high concentration of unconjugated estrogens in the fetus could place an emphasis on local expressions of sulfotransferases, not only in liver but also the male reproductive tract (Duanmu et al., 2006; Fietz et al., 2013; Miki et al., 2002; Mutembei et al., 2008; Schuler et al., 2018a, b; Wood, 2014).

In the adult male, circulating estrogen is low (Table 1), while T is high, as would be expected if spermatogenesis is to be preserved and masculinity maintained (Finkelstein et al., 2013; Zirkin and Papadopoulos, 2018). However, intratesticular E2 is elevated, as there is a shift toward sequestration within the reproductive tract, which results in substantial

increases in estrogen exiting the testis via rete testis fluid (see review: Cooke et al., 2017). In some species, such as the horse, the testis produces more estrone sulphate (5.4 μg/min) than testosterone (0.3 μg/min), much of which ends up in testicular lymph and rete testis fluid (Setchell and Cox, 1982). While the adult rat testis contributes about 21% of the blood E2 (de Jong et al., 1973), it contributes 100% to the rete testis fluid, where concentrations of E2 are reported to reach 510 pg/ml and average 249 pg/ml (Free and Jaffe, 1979). Although most of the E2 synthesis in the male reproductive system comes from the testis, the presence of aromatase has also been reported in the epithelium of efferent ductules and the epididymis (Carpino et al., 2004; Kim et al., 2008; Pereyra-Martinez et al., 2001; Rosati et al., 2020; Shayu and Rao, 2006; Swider-Al-Amawi et al., 2007). In addition, the external genitalia express aromatase (Jesmin et al., 2002).

The function of such high levels of estrogen in the lumen of the male tract remains unknown, but the efferent ductules have the highest expression of ESR1 of any tissue, male or female and presence of the receptor itself is absolutely required for fluid reabsorption physiology (Hess, 2018b; Hess and Cooke, 2018), while loss of luminal estrogen has no effect on the efferent ductules (Toda et al., 2008). Thus, it remains a mystery why such a high concentration of E2 would be found in the lumen of the rete testis in the first place, since it is not absolutely required for binding to the high concentration of ESR1 present downstream in the efferent ductule epithelium.

It is not known when, during development, the high concentration of E2 begins to appear in rete testis fluid. Estrogen is first produced in the testis during fetal life, with a significant increase in late gestation and the rete testis joins the developing mesonephric tubules around E15.5 in mice (Karl and Capel, 1995; Omotehara et al., 2020). However, seminiferous tubular lumens do not begin to open until PND10–15 in mice and rats (Auharek and de Franca, 2010), and full secretion of fluids is not achieved until PND30 in rats (Gondos and Berndston, 1993; Russell et al., 1989). Thus, fluid flow begins somewhere between 10–20 after birth and thereafter increases substantially. Therefore, a question arises regarding how testicular E2 might have direct influence on fetal development of the male reproductive tract? It is possible that prior to receiving fluid from the rete testis, the mesonephric tubules/early efferent ductules could transport circulating E2 into the lumen or synthesize E2 themselves. Mesonephric tubules form lumens as early as E11.5 (Omotehara et al., 2020) and thus are secretory. Because most studies of aromatase in the male have focused on the testis, only one paper has looked at the reproductive tract. Interestingly, they found high expression of aromatase mRNA in efferent ductules between PND7–14 but then a rapid drop in expression until there was no detectable presence at PND90 (Kim et al., 2008). This study should be extended into fetal development, as the mesonephros or the rete cord mesenchymal cells could be an interesting potential source of E2 for developmental influence over mesonephric tubule growth, fusion and branching.

For many years there has been a generally accepted hypothesis that Sertoli cells are the primary source of E2 production in the fetal and neonatal testis, while Leydig cells are the major source in the adult (Carreau et al., 1999; Carreau and Hess, 2010; Hess and Cooke, 2018; O'Donnell et al., 2001; Tsai-Morris et al., 1985). Some of the strongest support for this hypothesis came from ^{3}H-T conversion studies in fetal testis tissue in cultures

(Armstrong and Dorrington, 1977; Dorrington and Armstrong, 1975; Dorrington and Khan, 1993; Papadopoulos et al., 1986; Papadopoulos et al., 1987; Payne et al., 1987; Tsai-Morris et al., 1985; Valladares and Payne, 1979; Weniger and Zeis, 1983). Aromatase appears in the fetal mouse testis as early as E12.5 but around E17.5 shows a 20-fold increase in expression (Borday et al., 2013; Greco and Payne, 1994), although there was no E2 synthesis at E13.5. E2 synthesis was maximal in Sertoli cells from neonatal rats before the initiation of spermatogenesis and it was suggested that the release of E2 by Sertoli cells before puberty served to inhibit the synthesis of androgens by Leydig cells (Dorrington and Armstrong, 1979; Dorrington and Khan, 1993). A later report showed that endogenous E2 inhibited germ cell development in the fetal and neonatal mouse testis, which was thought to be acting through gonocyte expression of ESR2 (Delbes et al., 2004), but this could have also involved an inhibition of Leydig cell steroidogenic activity. After PND10, Sertoli cells are thought to produce very little E2 with or without FSH stimulation in the rat (Armstrong and Dorrington, 1977). In fact, Sertoli cell aromatase activity was found to be 7-fold higher in PND7–10 cells than at weaning and Leydig cells from animals older than PND15 were more capable of E2 synthesis than were Sertoli cells (Canick et al., 1979; Rommerts et al., 1982). However, one report showed no visible aromatase staining in the PND10 rat, but some staining of Leydig cells on PND18 (Lee et al., 2008). Nevertheless, in rodents the data appear to support the general statement that fetal and neonatal E2 is coming primarily from Sertoli cells, although Leydig cells appear to be capable of aromatase activity, if stimulated in culture, while the adult testis shows baseline Leydig cell activity (Borday et al., 2013; Greco and Payne, 1994; Warren et al., 1984; Weniger et al., 1993).

In humans the fetal and neonatal picture is not so clear. There are reports of low expressions of aromatase mRNA in the human fetal testis, as well as low E2 synthesis (Boukari et al., 2007; Tapanainen et al., 1989) and data showing E2 production at 8 mo postnatal and 3 yr that are nearly as high as that of adult testes (Inkster et al., 1995). Although aromatase immunostaining of Sertoli cells has been demonstrated at 14 wks prenatal (Boukari et al., 2007), in some studies Leydig and germ cells were also positive in the neonatal testis (Berensztein et al., 2006). However, by 35 wks of gestation, no staining could be found in the fetal testis, yet in the adult human, mRNA expression for aromatase was at least 4-fold higher than in the fetus (Boukari et al., 2007). Some studies have reported immunostaining for aromatase only in the adult interstitial area (Inkster et al., 1995). In the adult human testis, the major localization of aromatase has consistently been in Leydig cells (Brodie and Inkster, 1993; Payne et al., 1976), but germ cells, primarily elongating spermatids, also express the enzyme and they as well as sperm are capable of E2 synthesis and in some reports have a higher production than found in Leydig cells (Carreau et al., 2008; Carreau and Hess, 2010; Carreau et al., 2003; Guercio et al., 2020).

Aromatase in testicular germ cells was overlooked for decades, as it was generally accepted that Leydig cells were the primary site of E2 synthesis in the adult (Payne et al., 1976; Tsai-Morris et al., 1985; Valladares and Payne, 1979). However, this unanticipated discovery occurred when a very good aromatase antibody was used to localize the protein and the strong positive staining of elongating spermatids was not ignored and a curious graduate student asked the question: “Why is the center of the seminiferous tubule staining for aromatase?” (Hess et al., 1995; Nitta et al., 1993). Although we do not know why the

concentration of E2 in rete testis fluid is so incredibly high (Free and Jaffe, 1979), it now appears that the source of this estrogen is testicular spermatids and cytoplasmic droplets of luminal spermatozoa (Aquila et al., 2003; Aquila et al., 2002; Carpino et al., 2007; Carreau, 2007; Carreau and Hess, 2010; Janulis et al., 1996a; Janulis et al., 1998; Janulis et al., 1996b; Lambard and Carreau, 2005; Lambard et al., 2003; Lambard et al., 2004; Nitta et al., 1993; Rago et al., 2003).

5. Estrogen-associated knockout and transgenic models

Esr1 Knockout mouse

The development of gene knockout and transgenic technology has been crucial in the study of estrogen in the male reproductive system and in particular the *Esr1*KO mouse (Table 5). However, despite an abundance of reports on the adult phenotypes in the *Esr1*KO male, fetal and newborn effects have yet to be published. For a more in-depth coverage of the adult phenotype, the reader can examine several prior reviews (Cooke et al., 2017; Hess and Cooke, 2018; Hess et al., 2011; Hess et al., 2002; Joseph et al., 2011). In general, although the *Esr1*KO males were infertile, spermatogenesis appeared normal until puberty, when testis weight began to increase and progressive degeneration of the seminiferous epithelium occurred, while plasma LH and T were significantly elevated (Eddy et al., 1996; Lubahn et al., 1993). However, when germ cells from the *Esr1*KO testis were transplanted into wild-type testes of busulfan-treated mice, there was normal proliferation, differentiation and production of sperm with fertilizing capability (Eddy et al., 1996; Mahato et al., 2001). Thus, the major pathological problem was not the testis, but rather downstream defects in the reproductive tract (Eddy et al., 1996; Hess et al., 1997a). Subsequent studies found dramatic dilation of the efferent ductules and disruption of their physiological function (Hess et al., 1997a). Efferent ductule epithelium expresses an abundance of ESR1 (Hess et al., 1997b) and are responsible for reabsorption of nearly 90% of the luminal fluid (Clulow et al., 1998; Hansen et al., 1999), which contains sperm transported from the rete testis in a dilute seminiferous tubular fluid. This activity is controlled by ESR1 through transcriptional regulation of epithelial ion transporters, AQP (aquaporin) water channels, and Na^+/K^+-ATPase (Huang et al., 2006; Joseph et al., 2011; Lee et al., 2008; Lee et al., 2001; Ruz et al., 2006; Zhou et al., 2001). Loss of ESR1, as well as blockage of receptor function by a pure antiestrogen, resulted in the inhibition of fluid reabsorption, causing extreme dilation of the ductules, rete testis and eventually the seminiferous tubules, as fluid produced by the testis accumulated in the lumen and overwhelmed the only exit via the single distal efferent ductule that enters the initial segment epididymis (Cooke et al., 2017; Hess, 2014). The inhibition of fluid reabsorption in the efferent ducts, which involves the loss of a differentiated epithelial morphology (Hess and Cooke, 2018), as well as effects on epididymal fluid pH and osmolality, caused sperm tail coiling and premature acrosome reaction and loss of fertilizing capability (Joseph et al., 2010a; Joseph et al., 2010b). Epididymal abnormalities were also reported in the apical and clear cells and abnormal growth of epithelium of the initial segment in areas of the efferent ductules (Hess et al., 2000; Joseph et al., 2011). Thus, numerous physiological pathways are regulated by ESR1 in the adult male reproductive tract and accomplished even in the absence of E2, the natural receptor ligand (see discussion below).

The global deletion of *Esr1* raised the possibility that the adult phenotype was only a developmental phenomenon, which of course is important because the initial interest in estrogen in the male came from studies showing developmental anomalies following fetal and neonatal estrogen treatments. However, to receive NIH funding for these studies in the early 1990s there was a general requirement that there actually had to be a known function for estrogen in the adult male reproductive system. For that reason, all efforts were focused on deciphering the physiological role of ESR1 by comparing the *Esr1*KO phenotype to adult males treated with the potent antiestrogen, ICI 182,780 (Cho et al., 2003; Hess et al., 1997a; Oliveira et al., 2005; Oliveira et al., 2004; Oliveira et al., 2003; Oliveira et al., 2002; Zhou et al., 2001). The antiestrogen treatment confirmed that the adult male reproductive tract requires ESR1 for normal function of the rete testis and efferent ductules and maintenance of fluid reabsorption. Without this steroid receptor, luminal sperm showed abnormal maturation and morphology and inability to fertilize an egg. So, now the question remains - what is the developmental role of ESR1 in the male reproductive track?

Only a handful of studies have looked at developmental aspects of ESR1 in male reproduction (Bartlett et al., 2001; Delbes et al., 2005; Eddy et al., 1996; Goulding et al., 2010; Lee et al., 2008; Lee et al., 2009). Delbes et al. (2005) has been the only one to examine testes of the fetal *Esr1*KO male. They found an increase in Leydig cell volume in E13.5 fetuses and PND2 neonates and increased expression of genes responsible for T synthesis (Delbes et al., 2005), which demonstrated that the increase in T in the adult was already established in the fetus. However, this increase in androgen was incapable of making up for the loss of ESR1 downstream in the efferent ducts and epididymis. Their study also contained a rather important experiment related to DES, which showed that the fetal testis "is more sensitive to estrogens than the neonatal testis…", as DES treatment *in vitro* decreased basal T secretion in *Esr1*KO testes at E13.5 but had no effect on PND2 testes. However, in both fetal and neonatal wild-type testes, DES did decrease LH stimulation of T secretion, but had no effect in the *Esr1*KO. Thus, estrogen can have direct effects on the fetal and neonatal testes and decrease T synthesis, once there is LH production beginning around E16 in the mouse (O'Shaughnessy et al., 1998). Furthermore, this supports the idea that endogenous estrogen production by fetal Sertoli cells will have an inhibitory effect on fetal Leydig cells and T production and treatment with estrogens after E17.5 can greatly reduce androgen synthesis (Dorrington and Armstrong, 1975; Majdic et al., 1996).

Although treatment with the potent antiestrogen in adult rodents resulted in pathological changes nearly identical to those observed in the adult *Esr1*KO male (Cho et al., 2003; Hess et al., 1997a; Oliveira et al., 2005; Oliveira et al., 2004; Oliveira et al., 2003; Oliveira et al., 2002; Zhou et al., 2001), examination of PND10–20 tissues in *Esr1*KO mice confirmed the possibility that testicular and epididymal phenotypes were indeed developmental anomalies (Eddy et al., 1996; Lee et al., 2008; Lee et al., 2009). Between PND10–20, dilation of the rete testis had already begun, as well as luminal dilation in the seminiferous tubules. However, recently discovered archival images of *Esr1*KO testis revealed no differences in the seminiferous epithelium even closer to birth, as the lumens were closed and Sertoli and germ cells appeared normal at PND6 (Fig. 4).

The original report on *Esr1*KO mice did not find effects in the efferent ductules and epididymis at any age from PND10–60, although there was extreme dilation of the rete testis and seminiferous tubule lumens (Eddy et al., 1996). However, subsequent studies found dramatic dilation of efferent ductule lumens in the adult mice (Fig. 5) and reduction in epithelial height of greater than 50% (Hess et al., 2000; Lee et al., 2000; Lee et al., 2009). Thus, the question remained whether the long-term pathological changes were of developmental origin, especially since antiestrogen treatment with ICI 182,780 in adult male mice and rats had nearly identical effects in the testis, rete testis and efferent ductules (Cho et al., 2003; Lee et al., 2000; Oliveira et al., 2005; Oliveira et al., 2001; Oliveira et al., 2002; Zhou et al., 2001). There were also epididymal effects in both the *Esr1*KO mice and antiestrogen-treated mice and rats (Cho et al., 2003; Hess et al., 2000; Hess et al., 2011). However, these epididymal effects have been ignored in the literature, but in reality, provide rather strong evidence that ESR1 has a presence and activity in specific cell types of the epididymis and maybe during fetal development, as discussed in section 3.

In a subsequent study, PND10 and 18 were examined to determine if the observed adult phenotype, showing efferent ductule effects, was indeed present during postnatal development. Lee et al. (2008; 2009) found that as early as PND10 there was already luminal dilation of the ductules, as well as a reduction in epithelial height. They also found specific alteration in the expression of ion transporters necessary for the efferent ductules to perform their physiological function of fluid reabsorption. Recently discovered photos confirmed these published morphological findings and moved the efferent ductule dilation phenotype to as early as PND6 (Fig. 6). Thus, it appears that the male reproductive tract phenotype associated with the loss of ESR1 is an early developmental alteration, with early expansion of rete testis and efferent ductal lumens, with thinning of their respective epithelia (failure of differentiation). These neonatal morphological effects were accompanied by decreases in the expression of cystic fibrosis transmembrane conductance regulator (*Cftr*), chloride anion exchanger, *Slc26a3*, and *ATPase-α1* (Lee et al., 2008). Although the water channels AQP1 and 9 were not studied in neonatal *Esr1*KO males, it is likely that these will also be found decreased in expression, because they are expressed even in the fetal and neonatal efferent ductules (Fisher et al., 1999; Fisher et al., 1998) and have major roles in the movement of water from lumen to the interstitium (Verkman, 2002).

We do not know as yet if the rete testis expansion is due to the build-up of luminal fluid that is not reabsorbed by the efferent ductule epithelium or simply excessive proliferation of the epithelium. Further study of fetal and neonatal *Esr1*KO mice is required, as current publications are confusing and appear to support both hypotheses. For example, secretion by the seminiferous epithelium does not begin until approximately PND15 in the mouse (Auharek and de Franca, 2010; Gondos and Berndston, 1993; Nagano and Suzuki, 1976); therefore, the dilation of rete testis at or before birth due to accumulated seminiferous tubular fluid would not occur. Also, if fluid were to somehow leak into the rete testis lumen, an ESR1 mechanism seems missing because most studies have reported rete testis epithelium lacking ESR1 expression in both fetal and neonatal mice and rats (Nielsen et al., 2000; Sar and Welsch, 2000). However, during the developmental period, AR expression is abundant in rete testis epithelium (Magers et al., 2016; Williams et al., 2001a; You and Sar,

1998) and high doses of DES and other estrogens inhibit efferent ductule development (see section 6).

It was puzzling to find that perinatal treatments with DES or E2 induce the same expansion/dilation of rete testis lumen (Aceitero et al., 1998; Fisher et al., 1999; Fisher et al., 1998; Naito et al., 2014; Rivas et al., 2002; Rivas et al., 2003) as is found in the *Esr1*KO mouse and the anti-estrogen treated adult mice and rats (Cho et al., 2003; Hess, 2014; Hess et al., 1997a; Lee et al., 2000; Lee et al., 2009; Oliveira et al., 2002). Also, DES effects on prostate and seminal vesicles have been shown to be ESR1-dependent, but mediated through a permanent decrease in AR expression, as shown in DES-treated *Esr1*KO mice (Cederroth et al., 2007; Couse and Korach, 2004; Prins et al., 2001a; Walker et al., 2012). Therefore, DES effects on the rete testis must be further investigated, as there are no reports of ESR1 expression in the fetal mouse rete epithelium (Table 2). Table 6 lists prominent effects associated with efferent ducts and rete testis in the *Esr1*KO and antiestrogen treated rodents and other neonatal estrogen treatment models.

Neonatal high doses of DES work through its ability to down-regulate AR in the male reproductive system, along with a decrease in serum T, with no effect on ESR1 in efferent ductal epithelium, although changes in ESR1 expression in the cauda epididymis and initial part of the vas deferens have been observed (Williams et al., 2000). However, DES treatment may involve mechanisms that differ in the neonate compared to the adult male, because high doses of E2 in the adult result in nearly a total loss of both ESR1 and AR in the efferent duct epithelium (Oliveira et al., 2004) and down-regulation of AQP1(Fisher et al., 1998; Oliveira et al., 2005; Oliveira et al., 2004). These data are difficult to understand because castration alone removes efferent ductule AR expression but has no effect on the expression of AQP1, while causing a total loss of AQP9 (Oliveira et al., 2005). Although there is a loss of AR expression, castration also has no effect on ESR1. Interestingly, castration supplemented with either T or E2 rescued AQP9 and AR expressions (Oliveira et al., 2004). Thus, some genes in the male tract are responsive to both androgens and estrogens and some have both EREs and AREs (estrogen and androgen response elements) in their promoter regions, such as *Slc9a3* and *Slc9a3R1* (Snyder et al., 2009; Trepos-Pouplard et al., 2010; Yao et al., 2017). Both of these genes, as well as AQP9, are important for fluid reabsorption in the efferent ductules and it appears that their regulation requires some type of combination of AR and ESR1 expression and normal levels of only T, as E2 levels appear to be irrelevant for efferent duct development in the aromatase KO mouse (Robertson et al., 1999; Robertson et al., 2002; Toda et al., 2008).

Aromatase (Cyp19) Knockout mouse

The phenotype observed in the *Esr1*KO mouse (Hess et al., 1997a) pointed to the potential importance of “female hormones” in the male (Sharpe, 1997). We all assumed that E2 would be essential for development and differentiation of the male reproductive system, because aromatase is expressed in the testis from fetal life and an abundance of ESR1 and 2 are found throughout the system. It was assumed that the aromatase (*Cyp19*) knockout (AromKO) male mouse (Robertson et al., 1999; Robertson et al., 2002; Robertson et al., 2001) would have a phenotype comparable to that of the *Esr1*KO male (Table 5). However

surprisingly, the AromKO male showed normal development of testes, rete testis, efferent ductules and epididymis (O'Donnell et al., 2001; Robertson et al., 1999; Robertson et al., 2001; Toda et al., 2001) and normal to subfertility due to impaired mounting behavior (Honda et al., 1998). Testicular effects were observed only after aging and with phytoestrogen-free diets (Robertson et al., 2002). The only developmental reproductive effect reported was mild hypospadias (Cripps et al., 2019). Even with aging, the rete testis, efferent ductules and epididymis remained normal, suggesting normal physiology in the reproductive tract.

The AromKO phenotype remained perplexing for many years, as the loss of E2 synthesis in the male had very little effect on the reproductive system. This caused some scientists to ignore the extensive work with the *Esr1*KO male and treatments with a potent antiestrogen (Tables 5 and 6). However, this unexpected phenotype also raised many more questions. Answers to these questions finally began to arrive when Toda et al. (Toda et al., 2008) discovered that ESR1 expression remained abundant in efferent ductules of the AromKO. Thus, it appears that expression of ESR1 in the absence of E2 is capable of rescuing the male reproductive tract morphology and physiology. This finding was consistent with a previous study showing that ESR1 expression was constitutive in these ducts and present even after bilateral castration (Oliveira et al., 2004). Oliveira et al. also showed that only a high dose of E2 in the adult male was able to reduce ESR1 expression after castration, as treatments with low and high doses of T or dihydrotestosterone (DHT) showed no effect on ESR1. Several plausible explanations for lack of abnormalities in the AromKO male reproductive tract have been presented (Hess and Cooke, 2018; Toda et al., 2008) and include the following: a) ligand-independent transcriptional activation of the ESR1; b) binding of a testosterone metabolite, 5α-androstane- 3β-17β-diol, (although it has higher affinity for ESR2 (Kuiper et al., 1997; Weihua et al., 2002) but was found to be as effective as E2 in maintaining post-castration efferent ductal function (Picciarelli-Lima et al., 2006); c) maternal estrogen during fetal development and the postnatal period through the milk (Toda et al., 2008); and d) androgen regulation of key factors that contain both EREs and AREs, as previously mentioned. Although we do not have a full explanation for the AromKO, the requirement of ESR1 expression in the male tract still remains, as the presence of AR alone is not sufficient for development and differentiation of these tissues.

Other animal models

Several other animal models either directly or indirectly affect the expressions of *Esr1* or estrogen synthesis and metabolism and in some cases have phenotypes that resemble the *Esr1*KO male (Table 5). For example, in the CEARKO mouse there is conditional deletion of AR in the caput epididymal epithelium, which results in failure to develop the initial epididymal segment, but also 'induces' the expression of ESR1 throughout the caput epididymal epithelium (Krutskikh et al., 2011; O'Hara et al., 2011). It appears that this overexpression of ESR1 causes excessive fluid reabsorption leading to luminal occlusion and inflammation, fluid backup and dilation of rete testis and seminiferous tubules. As in the *Esr1*KO mouse, this results in long-term testicular atrophy. In the *Lgr4* mutant and *Lgr4*KO mice the major effect was in the efferent ductules rather than epididymis. In this model, ESR1 expression was decreased, but there was no effect on AR, and as expected the

phenotype also resembled that of the *Esr1*KO male, including decreased expression of AQP1, SLC9A3 and Na/K-ATPase α1 (Hoshii et al., 2007; Li et al., 2010; Mendive et al., 2006). In another model, conditional deletion of E2F4/5 transcription factors in only the efferent ductules, caused results that were also *Esr1*KO-like. Here again, there was a reduction in ESR1 expression and loss of AQP1 in the ductal epithelium (Danielian et al., 2016). Dilation of the rete testis in the E2F model was observed during the first week after birth, suggesting that this rete testis phenotype could be solely of efferent ductal origin. Although down regulation of ESR1 in the conditional *E2f4/5* model was small, it is possible that the effect was mediated through non-genomic mechanisms involving the membrane ESR1. A new transgenic model, in which only a nuclear ESR1 is found (NOER) and membrane ESR1 localization is lost, has a phenotype that is essentially identical to that of global deletion of *Esr1* (Nanjappa et al., 2016; Pedram et al., 2014). Thus, disruption of either the nuclear or membrane localization of ESR1 will inhibit development of the rete testis, efferent ductules and epididymis.

Summary of Animal Models

Disruption of ESR1 expression in efferent ductules and epididymis is clearly a major factor in development and differentiation of the male reproductive tract and in long-term production of sperm by the testis, which can lead to permanent infertility. However, loss of the natural ligand, E2, as shown in the AromKO mouse, does not equate to the *Esr1*KO and further study is required for a more complete understanding of the mechanisms involved with ligand-independent transcriptional activation of ESR1. Membrane ESR1 turns out to be as important as the nuclear localization of the receptor in efferent ductules and epididymis and its disruption requires further investigation to better understand how environmental factors could disrupt this pathway. Finally, the question of how fetal and neonatal DES exposure causes rete testis dilation has remained unanswered for many years. However, the *E2f4/5* knockout model demonstrated that disruption of only the efferent ductules (using the villin-cre, which is specific for efferent ducts in the male tract) can cause rete testis dilation, beginning as early as the first week after birth, which is identical to that observed in the *Esr1*KO mouse. Thus, neonatal DES treatment effects in the efferent ductules alone could induce rete testis dilation, but the mechanisms involved require further investigation, as the source of fluid accumulation in the lumen in the perinatal period is not yet known.

6. Effects of developmental exposures to exogenous estrogens

Estrogen-induced male reproductive tract abnormalities

In males, E2 production and action must be considered primarily on a local basis, i.e., E2 is produced locally to act locally. Although E2 in the general circulation can have systemic effects in males (e.g., on the hypothalamus-pituitary unit), it is rare for circulating E2 levels to be high enough to be capable of such actions. Within the male reproductive tract there are several well-documented sites of E2 production and action, as detailed elsewhere in this paper, and there is no shortage of substrate (testosterone) for the aromatase enzyme as the level of T in the testis and along the male reproductive tract is higher than anywhere else in the body. Indeed, it is unlikely that lack of substrate will be a determining factor in local E2 levels in the male reproductive tract, at least in a normal adult male. Thus, the most

important factor determining the local production of E2 will be the level of local expression of aromatase.

Interest in this area was awakened by evidence that prolonged treatment of pregnant women with DES in high doses (100–2300 μg/kg/day, depending on the stage of pregnancy (Palmer et al., 2009)) was associated with increased incidence of reproductive disorders in resulting sons; this included ~7-fold increase in incidence of urethral stenosis, ~4-fold increase in incidence of epididymal cysts and increased incidence of smaller testes and cryptorchidism, plus other evidence for penile and prostatic (squamous metaplasia) abnormalities (reviewed in: Toppari et al., 1996). However, a more recent follow-up cohort study of 1197 adult men who had been exposed chronically to DES in utero (Palmer et al., 2009), did not report such a high incidence of reproductive tract disorders compared with controls (n=1038), only finding significant (2- to 2.5-fold) increases in risk of cryptorchidism, epididymal cysts and inflammation of the testis.

Most of the experimental evidence for adverse effects of estrogens on the developing testis and male reproductive tract have involved exposure of rats and mice to high doses of the potent estrogen DES, and in some cases ethinyl estradiol (EE). Exposure has been either in pregnancy or immediately after birth in male neonates and, in general, comparable adverse changes have been reported in both developmental time periods. Before summarizing these effects, it is important to recognize some caveats. Administration of high doses of DES to pregnant rodents can cause adverse pregnancy outcomes, in particular reducing pup numbers and causing dystocia; the latter is particularly common in rats such that most studies of the effects of fetal exposure to DES on later reproductive structure and function in both males and females have used mice, which are less prone to dystocia. Because of this confounding effect, studies of DES effects on reproductive development in rats have generally used neonates.

High dose DES exposure of rats in neonatal life results in the following adverse changes in the reproductive tract: smaller testes (Atanassova et al., 2000; Atanassova et al., 1999; Goyal et al., 2003); reduced sperm count (Goyal et al., 2003) and fertility (Atanassova et al., 2000); fluid distension of the rete testis and efferent ducts (McKinnell et al., 2001; Rivas et al., 2002; Rivas et al., 2003) (Fig. 7); relative overgrowth of stromal tissue and relative undergrowth of epithelial tissue in the epididymis, vas deferens, prostate and seminal vesicles (Rivas et al., 2002; Rivas et al., 2003; Williams et al., 2001a; Williams et al., 2001b); smaller penis, hypospadias and reduced anogenital distance (Stewart et al., 2018). Some of these effects (e.g., prostate) are discussed in more detail elsewhere (Dobbs et al., 2019; Prins and Ho, 2010). Of all the DES-induced effects, perhaps the most dramatic is the gross distension of the rete and efferent ductules (Fig. 5), the latter change being comparable to what is also observed in *Esr1*KO mice (section 5).

Detailed analysis of the DES-induced abnormalities in the epididymis and vas has shown aberrant induction of epithelial ESR1 expression, coincident with gross reduction in epithelial cell height, a change that may indicate faulty cellular specification, i.e. epithelium appears flattened rather than columnar (Fig. 8). Consistent with this, the normally distinct boundary that demarcates the intense coiling of the distal cauda epididymis from the straight

duct of the vas deferens is blurred in rats exposed neonatally to high dose DES, such that in most animals the initial part of the vas deferens is coiled rather than straight (Atanassova et al., 2005a; Atanassova et al., 2001). The aberrant changes in stromal and epithelial tissue in the epididymis and vas deferens of DES-exposed rats also predisposes to inflammatory changes in the epididymis (Atanassova et al., 2005a; Miyaso et al., 2014), similar to those seen in the prostate (Bianco et al., 2006; Nelles et al., 2011; Prins et al., 2001b; Prins et al., 2006) and in testes of transgenic mice that overexpress aromatase (Li et al., 2006). Although virtually all of these abnormal changes were only induced after exposure to very high doses of DES, and were not inducible by high doses of weaker environmental estrogens such as bisphenol A and octylphenol (Atanassova et al., 2000; Rivas et al., 2002; Rivas et al., 2003; Williams et al., 2001a; Williams et al., 2001b), the conclusion from such studies is that locally produced estrogens, in physiological levels, probably play a fundamental role together with androgens in orchestrating normal development of the male reproductive tract from fetal life through to adulthood.

Considering that in rodent perinatal studies only doses of DES in excess of ~10μg/kg/day seem to result in adverse reproductive tract changes (Fielden et al., 2002; Goyal et al., 2003; LaRocca et al., 2011; Rivas et al., 2002; Williams et al., 2001a), this would imply that, based solely on estrogenicity, exposure of the developing male fetus to human-relevant levels of estrogenic environmental chemicals would not induce adverse reproductive development, as these are uniformly very weak estrogens. Consistent with this interpretation, rodent experimental studies have shown that perinatal exposure to bisphenol A (Goodman et al., 2009; LaRocca et al., 2011; Rivas et al., 2002; Williams et al., 2001a; Williams et al., 2001b), octylphenol or butylphenol (Haavisto et al., 2003; Rivas et al., 2002; Williams et al., 2001a; Williams et al., 2001b) at doses far in excess of normal human exposure are unable to replicate any of adverse effects of DES on male reproductive tract development. However, this does not exclude the possibility that a weak environmental estrogen might cause effects in man via a non-estrogen dependent mechanism, such has been mooted for bisphenol A (N'Tumba-Byn et al., 2012).

Considering that the doses of DES administered to pregnant women (100–2300 μg/kg/day) are equivalent to or higher than doses that cause adverse changes in perinatal rats, it is perhaps surprising that far greater adverse human effects were not reported. For example, administration of 50 or 100 μg DES/kg/day to pregnant rats resulted in 91% and 89–96.5% suppression of intratesticular testosterone levels (Haavisto et al., 2003; Mitchell et al., 2013), changes which would grossly impair normal masculinization in rats with a >50% incidence of hypospadias and cryptorchidism (van den Driesche et al., 2017). In reality, chronic DES exposure of the human male fetus resulted in only a modest, but significant, increase in incidence of cryptorchidism (Palmer et al., 2009), an increased incidence of epididymal cysts and small testes (Palmer et al., 2009; Wilcox et al., 1995) but no apparent impact on fertility (Wilcox et al., 1995). These findings could be interpreted as evidence that the human male fetus is relatively resistant to high DES exposure in pregnancy, which may be because the human fetus, unlike the rodent fetus, is normally exposed to very high endogenous E2 levels as part of normal pregnancy and is presumably adapted to this. Thus, E2 levels in human pregnancy range from ~500pg/ml in early pregnancy to 20,000pg/ml in

late pregnancy (Kuijper et al., 2013), whereas in rats and mice E2 levels are in the range 20–120 pg/ml throughout pregnancy (Camacho-Arroyo et al., 2018).

Mechanisms underlying estrogen-induced male reproductive tract abnormalities

There appear to be 3 changes that underlie DES-induced male reproductive tract abnormalities: elevated estrogen levels, reduced testosterone levels and reduced androgen receptor (AR) expression (Fig. 8).

Irrespective of whether DES exposure in rats has been before or after birth, the results show that high doses of DES markedly suppress testosterone production by the testis. Thus, administration of 50 or 100μg DES/kg/day to pregnant rats resulted in >90% suppression of fetal intratesticular testosterone levels (Haavisto et al., 2003; Mitchell et al., 2013), whereas treatment of neonatal rats from days 2–12 with ~370μg DES/kg/day resulted in ~75% suppression of plasma testosterone levels on postnatal day 15 (Rivas et al., 2002), a reduction that was still evident in adulthood (Atanassova et al., 1999; Goyal et al., 2003). Lower doses of DES caused proportionately smaller effects on testosterone levels; for example, 3.7μg DES/kg/day resulted in ~35% lowering of testosterone levels on day 15 and in adulthood. DES is equally able to suppress testosterone production by the fetal mouse testis (N'Tumba-Byn et al., 2012), but not by the human fetal testis, whether in culture (Habert et al., 2014; N'Tumba-Byn et al., 2012) or after xenografting into recipient mice (Mitchell et al., 2013); this species difference is discussed further below.

Mouse transgenic studies suggest that the adverse effects of DES/EE exposure on male reproductive tract development are mediated via ESR1. Thus, in *Esr1*KO mice, the adverse effects of perinatal DES exposure on development of the prostate (Couse and Korach, 2004) and seminal vesicles (Walker et al., 2012) do not occur, whereas they do in wild-type littermates. A more recent study (Nanjappa et al., 2019) suggested that selective knockout of membrane, but not cytosolic, ESR1 expression also protects against DES effects. From a physiological perspective, the evidence suggests that intratesticular E2 acts via ESR1 in fetal Leydig cells to negatively regulate Leydig cell size, steroidogenic enzyme expression and testosterone production, as all of these increase in *Esr1*KO mice (Delbes et al., 2005). This is reinforced by observations in the aromatase over-expressing mouse in which endogenous E2 levels are drastically elevated, as the males show grossly reduced testosterone levels, cryptorchidism and reproductive tract and prostate changes that are broadly similar to those observed in mice exposed developmentally to DES (Li et al., 2001).

The protective effect of *Esr1*KO against perinatal DES effects in mice has been studied in far more detail in female mice compared with male mice, but it is reasonable to presume that all of the adverse male reproductive tract effects of DES in mice are mediated via ESR1 (Couse and Korach, 2004). This is a pivotal observation, especially when assessing the translatability of mouse findings to the human. For example, as outlined above, one of the primary underlying causes for DES-induced adverse male reproductive effects in rodents is suppression of perinatal testicular testosterone production (Delbes et al., 2005; Haavisto et al., 2003; Mitchell et al., 2013; Rivas et al., 2002; Rivas et al., 2003), an effect presumed to be mediated via the expression of ESR1 in rodent Leydig cells (Tables 2, 3 and Mitchell et al., 2013). In contrast to the rodent testis, Leydig cells in the fetal human testis do not

express ESR1 and, accordingly, are resistant to the adverse steroidogenic effects of DES seen in rodents; this has been demonstrated in human fetal testis xenografts (Mitchell et al., 2013) and in human fetal testis cultures (reviewed in: Habert et al., 2014). From a physiological perspective, it perhaps makes sense that human fetal Leydig cells lack sensitivity to estrogen negative effects because of the 10-to100-fold higher circulating levels of E2 during human versus rodent pregnancy as outlined above.

In addition to drastically lowering endogenous testosterone levels, perinatal DES exposure in rats also reduces androgen action in target reproductive tract tissues by grossly suppressing AR protein expression (McKinnell et al., 2001; Williams et al., 2001a), an effect that occurs via increased proteosomic degradation of the AR protein rather than via altered AR gene expression (Woodham et al., 2003). Consequently, DES exposure effectively blocks normal androgen action by grossly reducing both the ligand (testosterone) and its target receptor. Considering the established pivotal role of androgen action in driving the normal development and function of the male reproductive tract, it is therefore unsurprising that exposure to high doses of DES causes multiple abnormalities of the male reproductive tract in rats and mice. Indeed, if neonatal rats are co-treated with high dose DES plus T, then virtually all of the adverse reproductive tract changes induced by DES alone are prevented (Atanassova et al., 2005a; McKinnell et al., 2001; Rivas et al., 2003). This poses the question as to whether DES is, effectively, acting just like an anti-androgen? The answer to this is probably no, because in neonatal rats treated with either a GnRH agonist, to reduce testosterone production, or the AR antagonist flutamide, to block androgen action, the adverse reproductive tract changes induced by DES are not reproduced, although there is a general retardation of reproductive development (McKinnell et al., 2001; Rivas et al., 2003; Williams et al., 2001a; Williams et al., 2001b). This suggests that for DES to induce adverse reproductive tract changes in the neonatal male rat, there has to be elevated estrogen action combined with lowered androgen action, namely alteration of the androgen-estrogen (A-E) balance.

7. The androgen-estrogen (A:E) balance

The notion of the A:E balance being important fits well with two fundamentally important factors. First, all cells/tissues that express AR in the male reproductive tract also express ESR1 and/or ESR2 at some stage of development (Tables 2–4). Second, testosterone supply is essential for estrogen action as it is the substrate for formation of E2 via the aromatase enzyme. Thus, differential regulation of the four key components (testosterone, AR, aromatase, ESR1/ESR2) that underpin the A:E balance would provide a simple but dynamic means of regulating reproductive tract development and function by locally modulating the balance in A:E action, via hormone levels or respective steroid receptor expression, according to local needs. It is also easy to see how gross distortion of the A:E balance (e.g., DES treatment, aromatase or ESR1 knockout) might disrupt normal development. But how do you prove that it is the A:E balance, as opposed to just hormone over- or under-exposure?

To elucidate if disturbance of the A:E balance is the mechanism underlying the adverse effects of high dose DES treatment in neonatal rats, researchers administered a DES dose that on its own caused no major adverse reproductive tract effects, but combined it with a

treatment to either reduce endogenous testosterone production (GnRH agonist) or to block endogenous androgen action using an androgen receptor antagonist (flutamide). These experimental manipulations of the A:E balance resulted in a spectrum of adverse reproductive effects similar to those induced by high dose DES on its own, although the effects were of smaller magnitude (Rivas et al., 2002). The same study also showed that when a high dose of bisphenol A was substituted for the low dose of DES in combination with the GnRH agonist, no significant adverse reproductive tract changes were found, implying that weak environmental estrogens such as bisphenol A are unable to disrupt the A:E balance sufficient to cause adverse effects in this experimental situation. Convincing though such studies are, they do not identify how the A:E balance actually operates nor what exactly constitutes a normal A:E balance.

If the A:E balance is critical for normal reproductive tract development in males, then we should be aware that this may also explain changes reported in other situations involving altered estrogen production or action. For example, in the AromKO mouse, which has negligible endogenous E2, testosterone levels are chronically elevated ~6-fold (Amano et al., 2017), resulting in gross disturbance of the A:E balance, this time in favor of androgens rather than estrogens as was the case in the DES studies outlined above, but did not result in rete testis and efferent duct abnormalities because the AR:ESR1 balance was still intact (Toda et al., 2008). In the *Esr1*KO mouse, which effectively blocks endogenous E2 action via this receptor (which is established to be the most important estrogen action pathway in the male reproductive tract), there is also 2- to 3-fold elevation of endogenous testosterone levels with unchanged E2 levels and zero estrogen action via ESR1 (Couse and Korach, 1999b; Delbes et al., 2005; Walker et al., 2012), again resulting in gross disturbance of the A:E balance. The similar distension of the efferent ducts and rete testis seen in *Esr1*KO mice (Hess, 2014; Hess and Cooke, 2018) and high dose DES-treated rats, could be interpreted as evidence that maintenance of a normal balance in A:E action underpins normal fluid resorption in these ducts, but also includes an AR:ESR1 balance (see section 5).

If the A:E balance is critical for normal male reproductive tract development and later function, how might it operate physiologically? Perhaps it is easiest to view it as a classical endocrine system involving negative feedback control as follows. Testosterone is produced by testicular Leydig cells, generating high levels within the testis and along the reproductive tract via the movement of fluid from the testis along the male reproductive tract. This movement of fluid will be very much higher in the mature than in the developing male. As T levels increase, a particular level is reached at which the aromatase enzyme, expressed within the testis in Leydig, Sertoli and germ cells according to age and stage of development (see section 5, Fig. 1, Table 1), is able to convert some of the T into E2, which then acts via ESR1 in Leydig cells to reduce testosterone production. As Sertoli cells appear to be the main source of aromatase in the perinatal testis (Carreau et al., 1999; Carreau and Hess, 2010; Hess and Cooke, 2018; O'Donnell et al., 2001; Tsai-Morris et al., 1985), this would constitute a paracrine short-loop negative feedback system. Thus, inappropriately high estrogen exposure, (e.g., perinatal DES treatment) plugs into this feedback system and grossly suppresses testosterone production, at least in rodents, as detailed above. However, what this scenario does not explain is why estrogen over-exposure also negatively regulates AR protein expression, which effectively blocks androgen action. One way of interpreting

this is that it is just another facet of regulating the balance between androgen-and estrogen-action and might be particularly important in the reproductive tract where it is not possible to negatively feedback control the supply of testosterone, so instead its ability to act via AR is regulated.

At puberty and beyond, the function of the reproductive tract changes dramatically, as germ cell numbers/types in the testis increase greatly leading up to the continual release of spermatozoa and their transport out of the testis and down the reproductive tract. The arrival of sperm demands new functions from the reproductive tract, as sperm need to undergo complex changes (e.g., membrane changes) associated with their functional maturation prior to their release from the body. The fact that sperm express aromatase and are immersed in testosterone in the surrounding fluid means that they can contribute to dynamic regulation of the A:E balance and can do so in a quantitative way as aromatase activity will increase in proportion to the number of sperm present. Thus, in theory, sperm can regulate their environment as they progress along the duct via manipulation of the relative action of testosterone and E2 on the neighboring ductal epithelium or underlying stroma.

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Abbreviations:

wt	weight
mo	months
ESR1	estrogen receptor 1
ESR2	estrogen receptor 2
AR	androgen receptor
T	testosterone
FSH	follicle stimulating hormone
E2	estradiol
ED	efferent ductule
KO	knockout mouse
ICI	antiestrogen ICI 182,780
DES	diethylstilbestrol
GnRH	gonadotrophin-releasing hormone antagonist
PPT	4,4’,4′-(4-Propyl-[1H] pyrazole- 1,3,5-triyl)
SLC9a3	sodium/hydrogen exchanger 3

AQP	aquaporin
CAR2 and 14	carbonic anhydrase 2 and 14
SLC4A4	sodium bicarbonate cotransporter
CFTR	cystic fibrosis transmembrane conductance regulator
GPER	G protein-coupled estrogen receptor 1
Cyp19	aromatase
LBD	ligand binding domain
DBD	DNA binding domain
HRE	hormone response element
ERE	estrogen response element
ARE	androgen response element
AF-1 and -2	activation functions 1 and 2 domains
DSP	daily sperm production
PND	postnatal day

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Highlights:

- Development of the male reproductive tract depends on fetal connections between mesonephric tubules, the Wolffian duct and rete testis cords
- Perinatal development depends on both androgen and estrogen receptor pathways
- Effects of *Esr1*KO on rete testis and efferent ducts begin during perinatal development
- High dose of estrogen (including DES) is required to produce an *Esr1*KO-like phenotype by reducing testosterone synthesis and androgen receptor protein
- An androgen:estrogen balance appears important for normal development/function

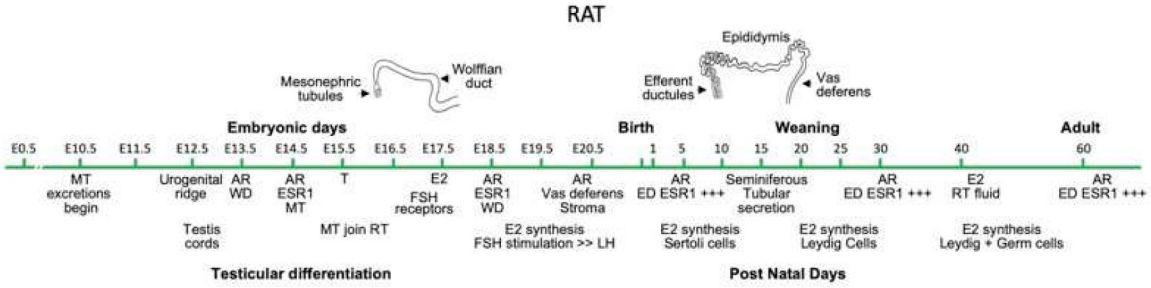

Fig 1.

Time course major events in development and differentiation of the rat male reproductive system. Although efferent ductules were emphysized for ESR1 expression, epididymal epithelium has positive expression in select cell types (see section 3). MT, mesonephric tubules; AR, androgen receptor; ESR, estrogen receptor; WD, Wolffian duct; T, testosterone; E2, estrogen; FSH, Follicle Stimulating Hormone; LH, Luteinizing Hormone; ED, efferent ductules; RT, rete testis. Adapted from Hinton & Avellar (2018).

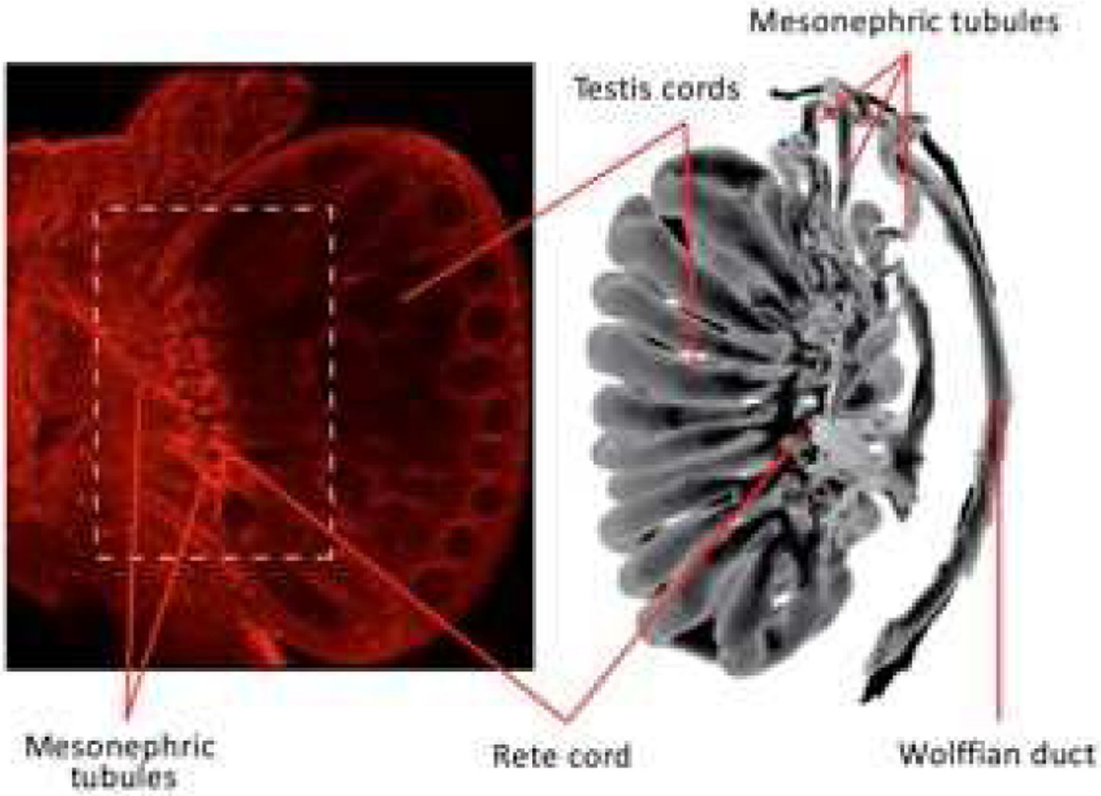

Fig 2.

Connections in the E15.5 mouse embryo showing testis, rete testis cord, mesonephric tubules and Wolffian duct. On the right is a three-dimensional model. Photos were modified from Combes et al. (2009) and De Mello Santos et al. (2019) and reproduced by permission of Wiley Press.

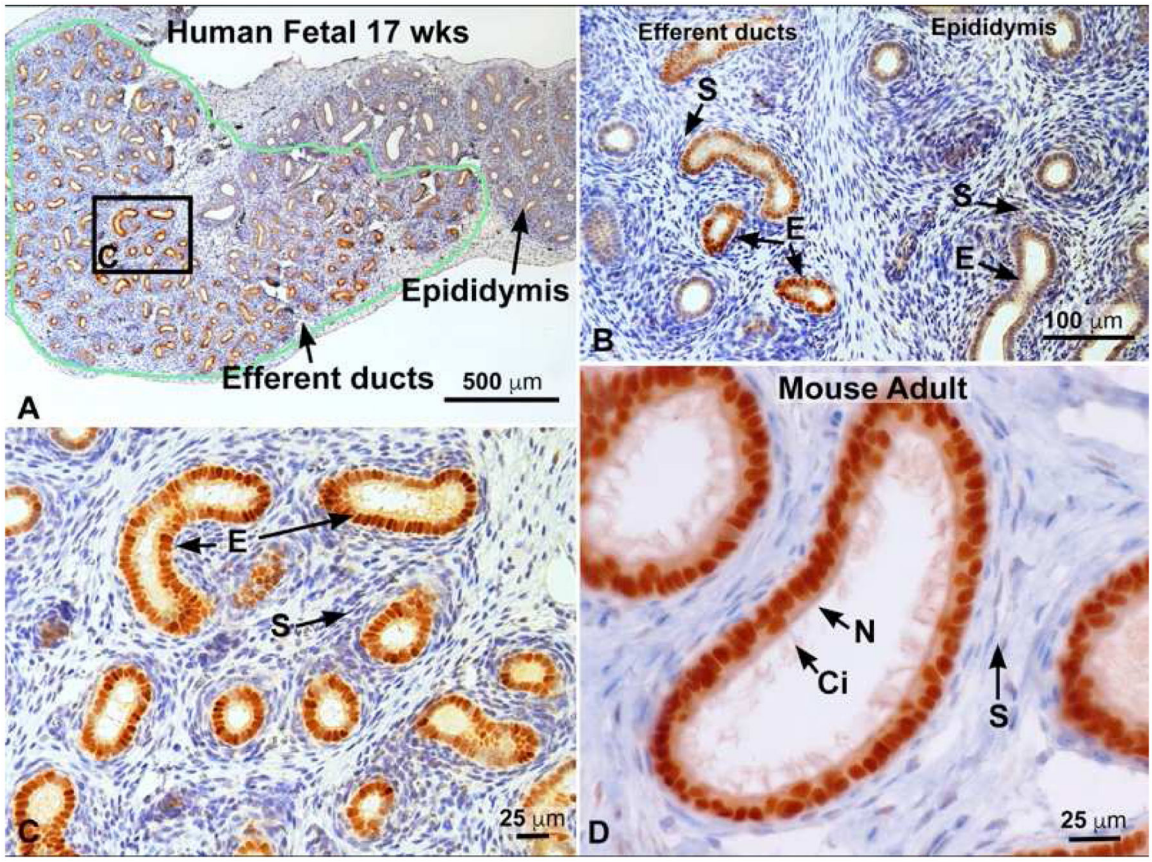

Fig. 3.

Estrogen receptor 1 (ESR1) immunostaining. A) 17 week fetal human efferent ductules in the head of the epididymis outlined in green and staining intensely for ESR1. To the right is the immature epididymal duct, which appears to have far less staining. B) Fetal 17 week human tissue showing efferent ductules to the left and epididymis to the right. Efferent duct stroma (S) is negative, but the epithelial cells (E) are strongly positive for ESR1, while epididymal epithelium is less positive and some stromal cells show slight staining. C) Higher magnification of area outlined in A. Efferent duct epithelial cells (E) are strongly positive for ESR1, but the stroma (S) is negative. D) Mouse adult efferent ductules showing intense staining for ESR1 in both nonciliated (N) resorptive epithelial cells, as well as the motile ciliated cells (Ci). Stromal (S) cells are mostly negative. Human fetal photos provided by Dr. Gerald Cunha, with approval by the Committee on Human Research at UCSF, IRB# 12–08813); Cunha et al. (2020).

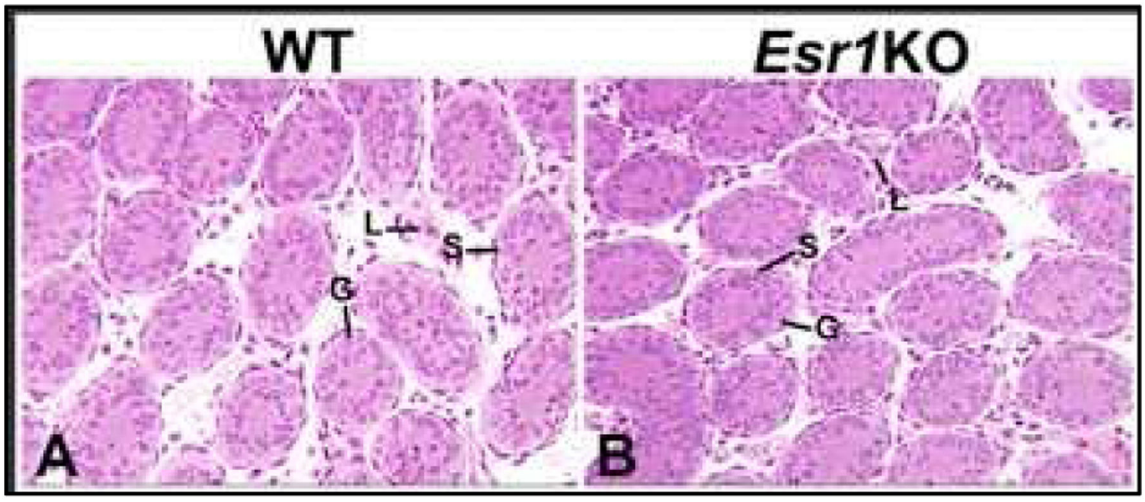

Fig 4.

Testes from PND6 wild-type (WT) and Esr1KO mice. A. WT seminiferous tubules are small in diameter with no lumen and an epithelium consisting of Sertoli and germ cells. Leydig cells are seen in the interstitial space. B. Seminiferous tubules and interstitial space in the Esr1KO mouse are similar to WT at this age and show no formation of a lumen.

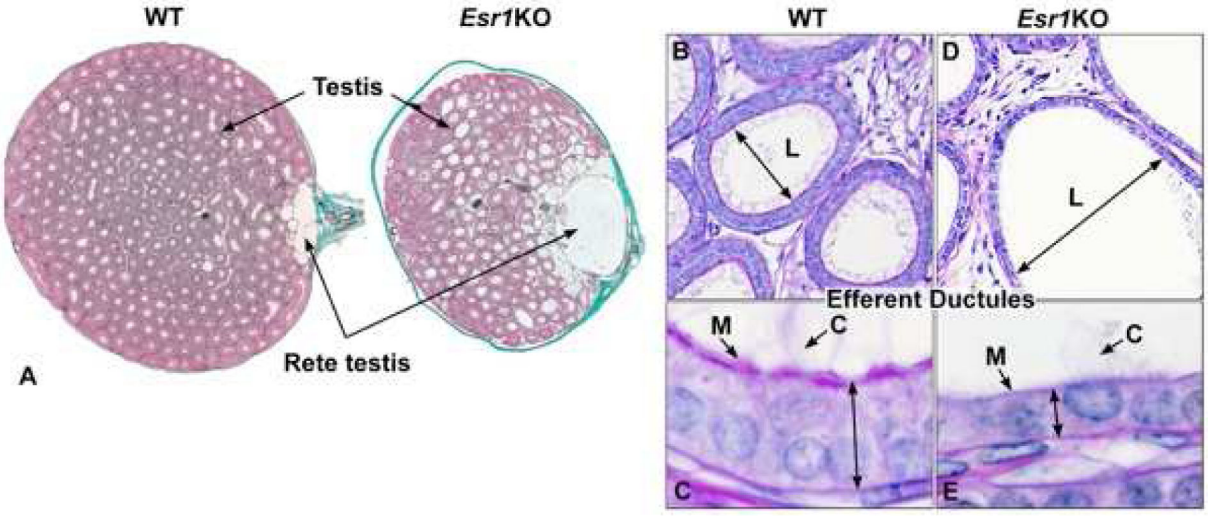

Fig 5.

Testis, rete testis and efferent ductules in the adult wild-type (WT) and Esr1KO mice. A) WT testis shows normal seminiferous tubular lumens with a small flattened rete testis where sperm exit to enter the efferent ductules. In contrast, the rete testis in an Esr1KO mouse shows excessive dilation. B) WT efferent ductules showing a normal luminal (L) diameter. C) Enlargement of the WT epithelium to illustrate normal height, long motile cilia (C) extending into the lumen and adjacent thick microvillus boarder (M) of the nonciliated, resorptive cells that are responsible for reabsorption of nearly 90% of the luminal fluids. D) Esr1KO efferent ductule, showing hyperdilation of the lumen (L). E) Higher magnification of the Esr1KO epithelium, which shows the dramatic decrease in height and loss of apical cytoplasm and microvilli (M) in the resorptive cells. Motile cilia (C) extend into the lumen, but appear to be fewer in number and shorter in length. Adapted from figures in Nanjappa et al. (2016) and Hess et al. (2011) with permission from Oxford University Press and John Wiley and Sons.

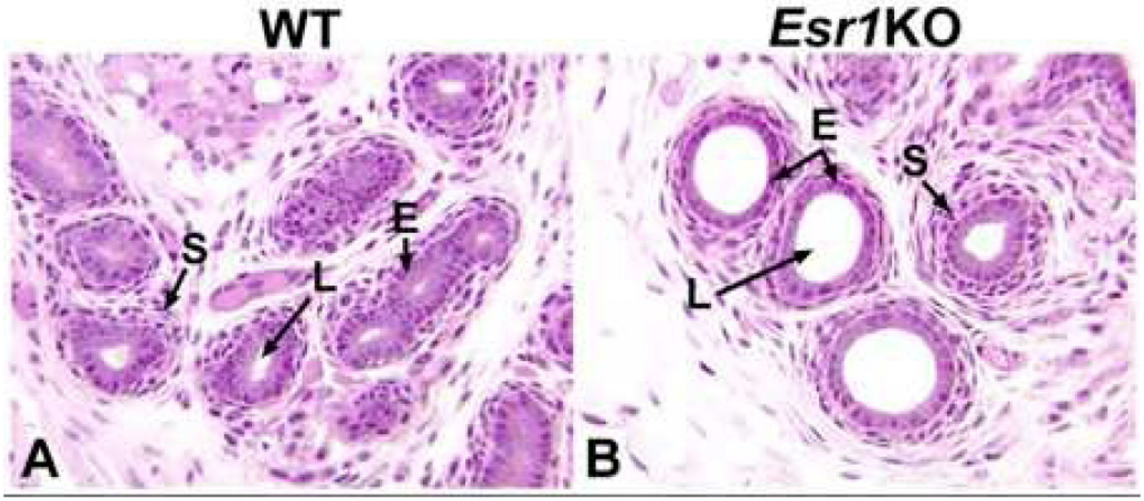

Fig 6.

Efferent ductules from PND 6 wild-type (WT) and Esr1KO mice. A) WT ductules have small diameters with tiny, but open lumens (L). The epithelium is short but taller than in the Esr1KO and the surrounding stromal cells make up about two layers. B) Efferent ductules from the Esr1KO mouse show strong luminal (L) dilation at this early age and the epithelium (E) is already decreased in height. The stromal cells appear normal.

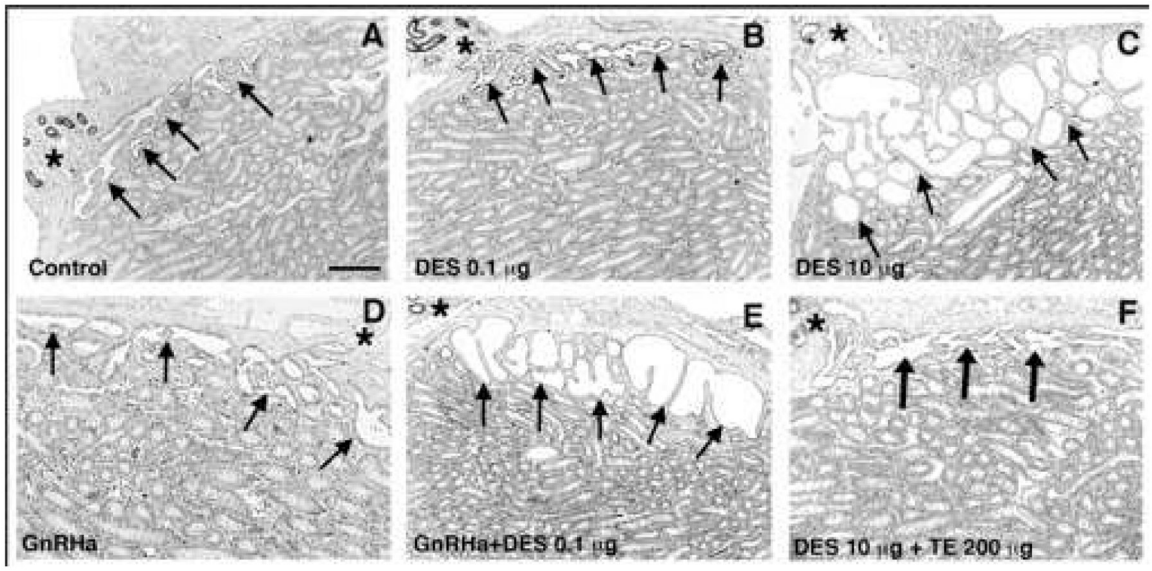

Fig. 7.

Effect of neonatal treatment with vehicle (control; A), a low dose (0.1 μg; B) or a high dose (10 μg; C) of diethylstilbestrol (DES), a GnRH antagonist (GnRHa; D), 0.1 μg DES plus GnRHa (E) or 10 μg DES + 200 μg testosterone esters (TE) (F) on the size of the rete testis lumen (arrows) at postnatal age 15 d. Note that all sections were obtained in the region in which the efferent ducts (asterisks) emerged from the rete along the testis capsule. Sections were immunostained for ESR1, hence the dark staining of nuclei of epithelial cells in the efferent ducts. Scale bar, 400 μm. Adapted from figures in Rivas et al (2002) and McKinnell et al (2001) with permission from Oxford University Press and John Wiley and Sons.

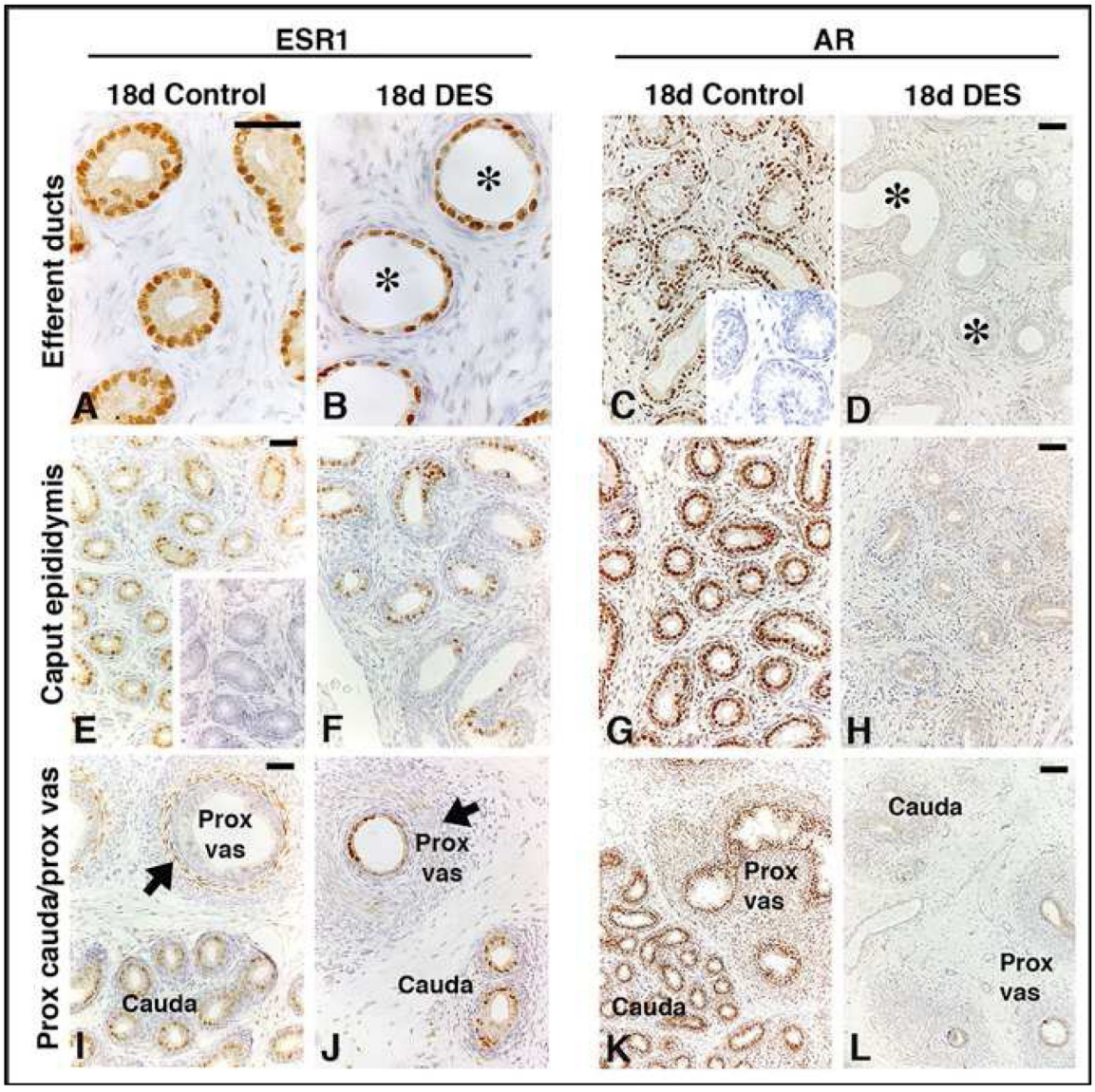

Fig. 8.

Effect of neonatal treatment of rats with vehicle (controls) or a high dose of diethylstilbestrol (DES; 10 μg) on immunoexpression of ESR1 (left-hand panels) and AR (right-hand panels) on postnatal day 18 in the efferent ductules (top row), caput epididymis (middle row) and proximal cauda/proximal vas deferens (bottom row). Note that, unlike in controls (A), epithelial expression of ESR1 in DES-treated rats extends into the proximal (J) regions of the vas deferens. Immunoexpression of ESR1 was unaffected in the efferent ducts (A vs. B), caput (E vs. F), and proximal cauda (I vs. J). Note also the reduction in height of the epithelium in the efferent ducts (A vs. B) and proximal vas deferens of DES-treated rats (I vs. J and K vs.L). DES also induced luminal distension of the efferent ducts (asterisks; A vs B). DES treatment profoundly reduced both epithelial and stromal expression of AR in the efferent ducts (C vs. D), caput epididymis (G vs. H) and proximal cauda and vas (K vs. L). Insets in panels C and E show negative controls in which the primary antibody was preabsorbed with the appropriate recombinant protein. Arrowheads point to stromal

immunoexpression of ESR1 in the proximal vas (I and J). Scale bars, 50 μm. Adapted from figures in Atanassova et al 2001 and McKinnell et al (2001) with permission from Oxford University Press and John Wiley and Sons.

Table 1.

Estradiol concentrations in the male

Age	Source	Species	Concentration	References
Fetal	Amniotic fluid	Human	64 pg/ml	*1*
	Blood	Mice	94 pg/ml	*2*
Neonatal PND 0	Umbilical cord artery	Human	5,000–12,000 pg/ml	*3*
Neonatal PND >60	Blood	Human	5–95 pg/ml	*3*
Neonatal PND 1	Blood	Rat	300–325 pg/ml	*4*
Neonatal PND 2	Blood	Rat	150–175 pg/ml	*5*
Neonatal PND 3–11	Blood	Rat	80–130 pg/ml	*6*
Adult	Blood	Human/monkey	3.6–145 pg/ml	*7*
	Blood	Rat	1.7–175 pg/ml	*8*
	Blood <	Mouse	11.8–30.0 pg/ml	*9*
	Testis	Human	5–20 ng/g	*10*
	Testis	Rat	4.5–751 pg/g	*11*
	Rete testis	Monkey	0.38 nmol/L	*12*
	Rete testis	Rat	249 pg/ml	*13*

References:

1-(Robinson et al., 1977)

2-(vom Saal et al., 1997)

3-(Anderson et al., 2010; Kuijper et al., 2013; Nagata et al., 2006)

4-(Döhler and Wuttke, 1975; Gorski et al., 1977)

5-(Döhler and Wuttke, 1975)

6-(Döhler and Wuttke, 1975; Gorski et al., 1977)

7-(Angsusingha et al., 1974; Bujan et al., 1993; Carreau et al., 2004; Cook et al., 1998; Dias et al., 2016; Doerr and Pirke, 1974; Finkelstein et al., 2013; Haug et al., 1974; Kley et al., 1976; Overpeck et al., 1978; Purvis et al., 1975; Waites and Einer-Jensen, 1974; Zhou et al., 2013)

8-(Butcher et al., 1974; Cook et al., 1998; de Jong et al., 1973; Döhler and Wuttke, 1975; Gorski et al., 1977; Hawkins et al., 1975; Jong et al., 1975; Södersten et al., 1974; Wichmann et al., 1984)

9-(Blomberg Jensen et al., 2013; Couse and Korach, 1999a; Taylor et al., 2020)

*10-*Carreau, 2004 #31563}

11-(Clarke and Pearl, 2014)

12-(Setchell et al., 1983)

13-(Free and Jaffe, 1979)

Table 2.

Fetal androgen and estrogen receptors in the human, rat and mouse male reproductive system

Tissue	Receptor	Presence	Species	FetalAge/Type of Study[1]	References
Whole testis	AR	−	Rat	<E15.5	(Majdic et al., 1995)
		+/−	Rat	E15	(You and Sar, 1998)
	ESR1	−	Human	23 wks 12–17 wks mRNA	(Gaskell et al., 2003)
		+	Human	23 wks mRNA	(Brandenberger et al., 1997)
		+++	Mouse	E14, E18.5 mRNA	(Cederroth et al., 2007; Mowa and Iwanaga, 2001)
	ESR2	+	Human	12–23 wks mRNA	(Brandenberger et al., 1997; Gaskell et al., 2003)
		+++	Mouse	E18.5 mRNA	(Cederroth et al., 2007)
Sertoli cell	AR	−	Rat	E17–20.5	(Majdic et al., 1995; Williams et al., 2001a)
		−	Mouse	E14	(Zhou et al., 1996)
	ESR1	−	Human	12–22 wks	(Gaskell et al., 2003; Shapiro et al., 2005)
		−	Rat	E17.5, 18.5, 20.5	(Fisher et al., 1997; Sar and Welsch, 2000; Saunders et al., 1998; Williams et al., 2001a)
		−	Rat	E19 mRNA	(Mowa and Iwanaga, 2001)
		−	Mouse	E13.5	(Nielsen et al., 2000)
		+/−	Mouse	E13–15 +; E17.5 +/−	(Greco et al., 1992)
	ESR2	+/−	Human	14–22 wks +; 35wks −	(Boukari et al., 2007; Gaskell et al., 2003; Takeyama et al., 2001)
		+/++	Rat	E16–20.5	(Saunders et al., 1998; van Pelt et al., 1999; Williams et al., 2001a)
		+/−	Rat	E19	(Mowa and Iwanaga, 2001)
Germ cell/gonocyt e	AR	+	Rat	E20.5	(Majdic et al., 1995)
		++	Mouse	E14–18	(Merlet et al., 2007; Zhou et al., 1996)
	ESR1	−	Human	12->21 wks	(Gaskell et al., 2003; Magers et al., 2016; Shapiro et al., 2005)
		−	Rat	E17.5, 18.5, 20.5	(Fisher et al., 1997; Sar and Welsch, 2000; Saunders et al., 1998)
		−/+	Rat	E14–17 mRNA −; E19 mRNA +/−	(Mowa and Iwanaga, 2001)
		−	Mouse	E13.5	(Nielsen et al., 2000)
		+	Mouse	E15	(Greco et al., 1992)
	ESR2	+	Human	14–22 wks	(Boukari et al., 2007; Gaskell et al., 2003; Takeyama et al., 2001)
		−	Human	35 wks	(Boukari et al., 2007)
		++	Rat	E16, 20.5	(Saunders et al., 1998; van Pelt et al., 1999)
		++	Rat	E17–19 mRNA	(Mowa and Iwanaga, 2001)
		+	Mouse	E16	(Jefferson et al., 2000)

Tissue	Receptor	Presence	Species	FetalAge/Type of Study[1]	References
Leydig cell (Interstitial)	AR	+/+++	Human	12 −22 wks; increasing	(Shapiro et al., 2005)
		−/+	Rat	E15	(You and Sar, 1998)
		++	Rat	E17–19.5 increasing	(Majdic et al., 1995; You and Sar, 1998)
		−	Mouse	E14–18	(Zhou et al., 1996)
	ESR	+++	Rat	E17 mRNA	(Mowa and Iwanaga, 2001)
		−	Mouse	E4–13 Receptor binding	(Holderegger and Keefer, 1986)
		+	Mouse	E14–17 Receptor binding	(Holderegger and Keefer, 1986; Stumpf et al., 1980)
	ESR1	++/+	Human	12–22 wks; decreasing	(Shapiro et al., 2005)
		−	Human	16–22 wks	(Gaskell et al., 2003; Magers et al., 2016)
		+++	Rat	E17.5–20.5	(Fisher et al., 1997; Sar and Welsch, 2000; Saunders et al., 1998; Saunders et al., 1997)
		++/+	Mouse	E13–15 decreasing	(Greco et al., 1993; Nielsen et al., 2000)
		+/−	Mouse	E17–19	(Greco et al., 1992)
	ESR2	++/+	Human	12–22 wks; decreasing	(Boukari et al., 2007; Gaskell et al., 2003; Shapiro et al., 2005)
		−	Human	35wks	(Boukari et al., 2007)
		++	Rat	E20.5	(Saunders et al., 1998)
		+/−	Rat	E17–19 mRNA	(Mowa and Iwanaga, 2001)
Peritubular myoid cell	AR	+/+++	Human	7->21 wks; increasing	(Magers et al., 2016; Shapiro et al., 2005)
		−	Rat	E15	(You and Sar, 1998)
		+	Rat	E17–20.5 increasing	(Majdic et al., 1995; You and Sar, 1998)
		+	Mouse	E15.5	(Merlet et al., 2007)
		−	Mouse	E14–18	(Zhou et al., 1996)
	ESR1	−	Human	16 wks	(Gaskell et al., 2003; Magers et al., 2016)
			Rat	E17.5, 18.5, 20.5	(Fisher et al., 1997; Sar and Welsch, 2000; Saunders et al., 1998)
		+/−	Mouse	E13.5	(Greco et al., 1992; Nielsen et al., 2000)
		++	Mouse	E17–19	(Greco et al., 1992)
	ESR2	++	Human	17 wks	(Boukari et al., 2007; Gaskell et al., 2003)
		−	Human	35 wks	(Boukari et al., 2007)
		++	Rat	E20.5	(Saunders et al., 1998)
Rete testis	AR	+++	Human	>21 wks	(Magers et al., 2016)
	ESR1	−	Human	>21 wks	(Magers et al., 2016)
		−	Mouse	E15.5 Epithelium −; stroma −	(Nielsen et al., 2000)
Mesonephros/efferent ducts	AR	++	Human	7->21 wks Epithelium ++; stroma ++ increasing	(Magers et al., 2016; Shapiro et al., 2005)
		+	Rat	E16 Receptor binding: epithelium	(Cooke et al., 1991a)

Tissue	Receptor	Presence	Species	FetalAge/Type of Study[1]	References
		+	Rat	E14 Epithelium +; stroma +/−	(Bentvelsen et al., 1995; You and Sar, 1998)
		++	Rat	E17–19.5 Epithelium ++; stroma +	(Majdic et al., 1995; You and Sar, 1998)
		+	Rat	E17.5, 18.5 stroma	(Fisher et al., 1997)
		++	Mouse	E16–19 Receptor binding	(Cooke et al., 1991a)
	ESR	++	Mouse	E16 Receptor binding: epithelium +++; stroma ++	(Cooke et al., 1991b)
	ESR1	−	Human	8–9 wks Epithelium −; stroma −	(Cunha et al., 2020)
		+++	Human	12->21 wks Epithelium +++; stroma −	(Cunha et al., 2020; Gaskell et al., 2003; Magers et al., 2016)
		−	Rat	E17.5–18.5 Epithelium −; stroma +++	(Fisher et al., 1997)
		+++	Rat	E17 mRNA Epithelium +++; stroma +	(Mowa and Iwanaga, 2001)
		−	Mouse	E11.5 Epithelium −; stroma −/+	(Nielsen et al., 2000)
		+/++	Mouse	E13-E15; increasing	(Greco et al., 1992)
		+/−	Mouse	E15.5 Epithelium +/−; stroma −	(Nielsen et al., 2000)
	ESR2	−	Human	8–9 wks Epithelium −; stroma −	(Cunha et al., 2020)
		+	Human	12–20 wks Epithelium +; stroma +	(Cunha et al., 2020)
		−	Rat	E17–19 mRNA Epithelium stroma +/−	(Mowa and Iwanaga, 2001)
Wolffian duct/Epidid ymis	AR	++	Human	7->21 wks Epithelium ++; stroma ++	(Magers et al., 2016; Shapiro et al., 2005)
		−/+	Rat	E14–16.5 Epithelium −; stroma +	(Bentvelsen et al., 1995; You and Sar, 1998)
		+	Rat	E17–18 Epithelium caput +	(Majdic et al., 1995)
		−	Mouse	E14–16 Receptor binding: epithelium −; stroma ++	(Cooke et al., 1991a)
		++	Mouse	E19-PND 0 Receptor binding: epithelium ++/++	(Cooke et al., 1991a)
	ESR	−	Mouse	E4–10 Receptor binding: epithelium −; stroma −	(Holderegger and Keefer, 1986)
		−/++	Mouse	E13–17 Receptor binding: epithelium −; stroma +/++	(Holderegger and Keefer, 1986)
		−/+++	Mouse	E16 Receptor binding: epithelium −; stroma +++	(Cooke et al., 1991b; Stumpf et al., 1980)
		+	Mouse	E19 Receptor binding: epithelium +	(Cooke et al., 1991b)
	ESR1	−	Human	8–9 wks Caput −; cauda −	(Cunha et al., 2020)
		+	Human	12 wks Caput, cauda epithelium +; stroma +	(Cunha et al., 2020)
		++/−	Human	7–22 wks Caput epithelium + +; Cauda − decreasing	(Cunha et al., 2020; Shapiro et al., 2005)

Tissue	Receptor	Presence	Species	FetalAge/Type of Study[1]	References
		−	Rat	>21 wks Epithelium	(Magers et al., 2016)
		−/++	Mouse	E14–19 mRNA Epithelium −; stroma ++/+++	(Mowa and Iwanaga, 2001)
		+/++	Mouse	E13–15 Increasing	(Greco et al., 1992; Nielsen et al., 2000)
		+/−	Mouse	E17–19 Decreasing	(Greco et al., 1992)
	ESR2	++	Human	7–22 wks Epithelium ++; stroma ++	(Shapiro et al., 2005; Takeyama et al., 2001)
		−	Human	8–9 wks Cauda	(Cunha et al., 2020)
		+	Human	12–20 wks Cauda	(Cunha et al., 2020)
		−	Rat	E14–19 mRNA Epithelium −; stroma +/−	(Mowa and Iwanaga, 2001)
Vas deferens	AR	++	Human	>21 wks Epithelium ++; stroma +; smooth muscle −	(Magers et al., 2016)
		+	Rat	E20 Stroma	(Bentvelsen et al., 1995)
		+	Mouse	E19-PND 0 Receptor binding	(Cooke et al., 1991a)
	ESR1	−	Human	>21 wks	(Magers et al., 2016)
		−/++	Rat	E14 mRNA Epithelium − ; stroma ++	(Mowa and Iwanaga, 2001)
		−	Mouse	E13.5	(Nielsen et al., 2000)
	ESR2	−	Rat	E14–19 mRNA Epithelium −; stroma +/−	(Mowa and Iwanaga, 2001)

[1] Immunohistochemistry unless otherwise indicated; mRNA can be isolated RNA or *insitu* hybridization; Receptor binding can be autoradiography or biochemical assay binding

Table 3.

Neonatal androgen and estrogen receptors in the human, rat and mouse male reproductive system

Tissue	Receptor	Presence	Species	Neonatal Age/Type of Study[1]	References
Whole testis	ESR1	−	Human	12–60 mo: mRNA	(Berensztein et al., 2006)
		+++	Mouse	PND 1,5,12,19 mRNA	(Jefferson et al., 2000)
	ESR2	+++	Human	12–60 mo: mRNA	(Berensztein et al., 2006)
		+++/+	Mouse	PND 1,5 +++; PND 12,19 + mRNA	(Jefferson et al., 2000)
Sertoli cell	AR	−	Human	<22d-7 mo	(Berensztein et al., 2006)
		−	Rat	PND 2	(You and Sar, 1998)
		+/++	Rat	PND 5–14 Increasing	(Bremner et al., 1994; Majdic et al., 1995; You and Sar, 1998)
		++	Rat	PND 15–18	(McKinnell et al., 2001; Suarez-Quian et al., 1996; Williams et al., 2001 a)
		−	Mouse	PND 2	(Tan et al., 2005)
		++	Mouse	PND 10–12	(De Gendt et al., 2009; Denolet et al., 2006; Tan et al., 2005)
	ESR1	−	Human	<22 wks	(Berensztein et al., 2006)
		−	Rat	PND 1–10	(Fisher et al., 1997; Sar and Welsch, 2000)
		++	Rat	PND 15 mRNA Primary culture	(Lucas et al., 2008)
		+	Rat	PND 15	(Lucas et al., 2008)
		−	Mouse	PND 1–19	(Jefferson et al., 2000; Nielsen et al., 2000)
	ESR2	+++/+	Human	<22d-7 mo decreasing	(Berensztein et al., 2006)
		+/++	Rat	PND 3–15	(Lucas et al., 2008; Saunders et al., 1998; van Pelt et al., 1999)
		++	Rat	PND 15 mRNA	(Lucas et al., 2008)
Germ cells/ gonocytes	AR	−	Human	<22d-7 mo decreasing	(Berensztein et al., 2006)
		−	Rat	PND 3–7	(Majdic et al., 1995)
	ESR1	−	Human	<22d-7 mo	(Berensztein et al., 2006)
		+	Rat	PND 7 mRNA	(Mowa and Iwanaga, 2001)
		−	Rat	PND 1–10	(Fisher et al., 1997; Sar and Welsch, 2000)
		−	Mouse	PND 1–12	(Jefferson et al., 2000; Nielsen et al., 2000; Sato et al., 1994)
	ESR2	+++	Human	<22d-7 mo	(Berensztein et al., 2006)
		++/+++	Rat	PND 3–4	(Saunders et al., 1998; van Pelt et al., 1999)
		++	Rat	PND 7 mRNA	(Mowa and Iwanaga, 2001)
		+/++	Mouse	PND 5 +; PND 12 ++	(Jefferson et al., 2000)
Leydig cells/ Interstitial	AR	+	Human	<22d-7 mo	(Berensztein et al., 2006)
		+/++	Rat	PND 2–18 increasing	(Bremner et al., 1994; McKinnell et al., 2001; Tan et al., 2005; Williams et al., 2001a; You and Sar, 1998)
		++	Mouse	PND 2–12	(De Gendt et al., 2009; Denolet et al., 2006; Tan et al., 2005)

Tissue	Receptor	Presence	Species	Neonatal Age/Type of Study[1]	References
	ESR	+	Mouse	PND 1 Receptor binding	(Holderegger and Keefer, 1986)
	ESR1	+/−	Human	<22d-7 mo	(Berensztein et al., 2006; Mitchell et al., 2013)
		++	Rat	PND 7 mRNA	(Mowa and Iwanaga, 2001)
		++	Rat	PND 1–10	(Fisher et al., 1997; Nielsen et al., 2000; Sar and Welsch, 2000)
		+	Mouse	PND 0–12	(Jefferson et al., 2000; Nielsen et al., 2000; Sato et al., 1994)
	ESR2	+++/+	Human	<22d-7 mo	(Berensztein et al., 2006)
		++	Rat	PND 3,10	(Saunders et al., 1998)
		−	Rat	PND 4, 11	(van Pelt et al., 1999)
		+/−	Rat	PND 7	(Mowa and Iwanaga, 2001)
Peritubular myoid	AR	+++	Human	<22d-7 mo decreasing	(Berensztein et al., 2006)
		+/++	Rat	PND 2–18 increasing	(Bremner et al., 1994; McKinnell et al., 2001; Suarez-Quian et al., 1996; Williams et al., 2001a; You and Sar, 1998)
		++	Mouse	PND 2–12	(De Gendt et al., 2009; Denolet et al., 2006; Tan et al., 2005)
	ESR1	−/+	Human	<22d-7mo	(Berensztein et al., 2006)
		−	Rat	PND 1–10	(Fisher et al., 1997; Sar and Welsch, 2000)
		+	Mouse	PND 0	(Nielsen et al., 2000)
		−	Mouse	PND 1–12	(Jefferson et al., 2000)
	ESR2	+++/+	Human	<22d-7mo decreasing	(Berensztein et al., 2006)
		++	Rat	PND 3,10	(Saunders et al., 1998)
		−	Rat	PND 4, 11	(van Pelt et al., 1999)
Rete testis	AR	+++	Rat	PND 5, 18	(Williams et al., 2001a; You and Sar, 1998)
	ESR1	+	Rat	PND 4–10 Epithelium +; stroma +/−	(Fisher et al., 1997)
		−	Rat	PND 0–2	(Nielsen et al., 2000; Sar and Welsch, 2000)
		−	Mouse	PND 0	(Nielsen et al., 2000)
Efferent ducts	AR	+++/++	Rat	PND 15–18 Epithelium +++/++; stroma −	(McKinnell et al., 2001; Rivas et al., 2002; Williams et al., 2001a)
		++	Mouse	PND 0–10 Receptor binding: epithelium ++; stroma ++	(Cooke et al., 1991a)
		+++	Mouse	PND 11	(O'Hara et al., 2011)
	ESR1	+++/−	Rat	PND 0–15 Epithelium +++; stroma −	(Atanassova et al., 2001; Fisher et al., 1997; Nielsen et al., 2000; Rivas et al., 2002; Rivas et al., 2003; Williams et al., 2000)
		+++/−	Rat	PND 7 mRNA Epithelium +++; stroma +/−	(Mowa and Iwanaga, 2001)
		+++/−	Mouse	PND 0–10 Epithelium +++; stroma −	(Lee et al., 2008; Nielsen et al., 2000; Sar and Welsch, 2000; Sato et al., 1994)
	ESR2	++/+	Rat	PND 10 Epithelium ++/+; stroma −/+	(Williams et al., 2000)

Tissue	Receptor	Presence	Species	Neonatal Age/Type of Study[1]	References
		−/+	Rat	PND 7 mRNA Epithelium −; stroma +/−	(Mowa and Iwanaga, 2001)
		+++/++	Mouse	PND 1–10 Epithelium +++/++; stroma +++	(Lee et al., 2008; Sar and Welsch, 2000)
Epididymis	AR	+	Rat	PND 1, 10 Epithelium −/+; stromal + shifting to Epithelium	(You and Sar, 1998)
		+	Rat	PND 5,10 Epithelium +; stroma +	(Bentvelsen et al., 1995)
		+++	Rat	PND 18 Epithelium +++; stroma +/−	(McKinnell et al., 2001; Williams et al., 2001 a)
		+++	Mouse	PND 11	(Hoshii et al., 2007; O'Hara et al., 2011)
		++	Mouse	PND 0–10 Receptor binding: epithelium ++; stroma ++	(Cooke et al., 1991a)
	ESR	−	Mouse	PND 1 Receptor binding: epithelium −; stroma +++	(Holderegger and Keefer, 1986)
		+	Mouse	PND 0–10 Receptor binding: epithelium +; stroma +	(Cooke et al., 1991a)
		+	Mouse	PND 0–10 Initial segment > caput-cauda	(Cooke et al., 1991a)
	ESR1	++/−	Rat	PND 1 −8 Initial segment Epithelium ++/−; stroma +/−	(Fisher et al., 1997; Sar and Welsch, 2000)
			Rat	PND 1 −8 Caput to cauda Epithelium −; stroma +/−	(Fisher et al., 1997; Sar and Welsch, 2000)
		−	Rat	PND 10 Epithelium −; stroma −	(Atanassova et al., 2001; Williams et al., 2000)
		++/−	Rat	PND 18 Epithelium +/−; stroma −/++ region specific	(Atanassova et al., 2001)
		+++	Mouse	PND 1–12 mRNA; strongest d12	(Jefferson et al., 2000)
		++/+	Mouse	PND 0–12 Epithelium ++/+; stroma +/−	(Hoshii et al., 2007; Jefferson et al., 2000; Nielsen et al., 2000; Sato et al., 1994)
	ESR2	++/−	Rat	Epithelium ++/−, gradient caput > cauda; stroma −/+	(Sar and Welsch, 2000; Williams et al., 2000)
		+/−	Mouse	mRNA	(Jefferson et al., 2000)
		++/−	Mouse	PND 7−12 Epithelium ++/−; stroma ++	(Hoshii et al., 2007; Jefferson et al., 2000)
Vas deferens	AR	+	Rat	PND 2 Epithelium +; stroma +; PND 5 basal cells −	(Bentvelsen et al., 1995)
		+++	Rat	PND 18 Epithelium +++; stroma +/−	(McKinnell et al., 2001; Williams et al., 2001a)
		++	Mouse	PND 0–10 Receptor binding: epithelium ++; stroma ++	(Cooke et al., 1991a)
	ESR	+	Mouse	PND 0–10 Receptor binding: epithelium +; stroma +	(Cooke et al., 1991a)
	ESR1	−	Rat	PND 10 Epithelium −; stroma −	(Atanassova et al., 2001; Williams et al., 2000)
		−	Rat	PND 18 Epithelium −; stroma +++	(Atanassova et al., 2001; Rivas et al., 2003)
		+++	Mouse	PND 1–19 mRNA	(Jefferson et al., 2000)
		−	Mouse	PND 0–12 Epithelium −; stroma −/+	(Nielsen et al., 2000; Sato et al., 1994)

Tissue	Receptor	Presence	Species	Neonatal Age/Type of Study[1]	References
	ESR2	−	Mouse	PND 1–19 mRNA	(Jefferson et al., 2000)

[1] Immunohistochemistry unless otherwise indicated; mRNA can be isolated RNA or *insitu* hybridization; Receptor binding can be autoradiography or biochemical assay binding; PND, postnatal day; mo, months

Table 4.

Early puberty and adult androgen and estrogen receptors in the human, rat and mouse male reproductive system

Tissue	Receptor	Presence[1]	Species	Type of Study[1]	References
Whole testis	ESR	+	Human	Receptor binding	(Murphy et al., 1980)
	ESR1	+	Human	mRNA	(Denger et al., 2001)
		+	Mouse	mRNA	(Jefferson et al., 2000)
	ESR2	+	Human	mRNA	(Denger et al., 2001; Saunders et al., 2002)
Sertoli cell	AR	+++	Human	IM	(Iwamura et al., 1994; Kimura et al., 1993; Pelletier, 2000)
		++	Rat	mRNA	(Kumar et al., 2018)
		++/+++	Rat	Stage-dependent	(Bremner et al., 1994; Figueiredo et al., 2016; O'Hara and Smith, 2015; Oliveira et al., 2003; Pelletier, 2000; Pilutin et al., 2014; Suarez-Quian et al., 1996; Takeda et al., 1990; Tan et al., 2005; Turner et al., 2001; Vornberger et al., 1994; Williams et al., 2001a; Zhu et al., 2000)
		+++	Mouse	Stage-dependent	(Krutskikh et al., 2011; Schauwaers et al., 2007; Smith et al., 2015; Welsh et al., 2010; Zhou et al., 2002; Zhou et al., 1996)
	ESR1	−	Human	IM	(Fietz et al., 2014; Mäkinen et al., 2001; Pelletier and El-Alfy, 2000; Saunders et al., 2001; Saunders et al., 2003)
		−	Rat	mRNA	(Kumar et al., 2018; Mowa and Iwanaga, 2001)
		++	Rat	mRNA	(Lucas et al., 2008)
		++	Rat	IM	(Lucas et al., 2008; Menad et al., 2017; Mizuno et al., 2011)
		−	Rat	IM	(Gur and Aktas, 2020; Pilutin et al., 2014)
		−	Mouse	IM	(Cooke et al., 2017; Jensen, 2014; Zhou et al., 2002)
	ESR2	++/−	Human	IM	(Fietz et al., 2014; Gibson and Saunders, 2012; Mäkinen et al., 2001; Pelletier and El-Alfy, 2000; Saunders et al., 2002; Saunders et al., 2003)
		+/++	Rat	IM	(Lucas et al., 2008; Oliveira et al., 2003; Pilutin et al., 2014; Turner et al., 2001; van Pelt et al., 1999)
		+	Rat	mRNA	(Kumar et al., 2018; Lucas et al., 2008)
		−	Rat	IM	(Gur and Aktas, 2020; Menad et al., 2017)
		++	Mouse	IM	(Cooke et al., 2017; De Gendt et al., 2004; Zhou et al., 2002)
		−	Mouse	IM	(Jensen, 2014)
Germ cells/sperm	AR	++	Human	IM	(Kimura et al., 1993)
		−	Human	IM	(Iwamura et al., 1994)
		−	Rat	IM	(O'Hara and Smith, 2015; Oliveira et al., 2003; Takeda et al., 1990)
		+	Rat	IM Spermatids	(Suarez-Quian et al., 1996; Vornberger et al., 1994)
		−	Mouse	IM	(Zhou et al., 2002)
		++	Mouse	IM Spermatogonia	(Zhou et al., 1996)

Tissue	Receptor	Presence[1]	Species	Type of Study[1]	References
	ESR1	−	Human	IM	(Mäkinen et al., 2001; Pelletier and El-Alfy, 2000; Saunders et al., 2001; Saunders et al., 2003)
		++/−	Human	IM	(Fietz et al., 2016; Fietz et al., 2014)
		+	Human	mRNA sperm	(Lambard et al., 2004)
		++	Rat	IM Spermatids	(Gur and Aktas, 2020; Huang et al., 2016; Lucas et al., 2008; Menad et al., 2017; Mizuno et al., 2011; Pilutin et al., 2014)
		+/−	Rat	Germ cell mRNA	(Kumar et al., 2018; Mowa and Iwanaga, 2001)
		−	Mouse	IM	(Cooke et al., 2017; Zhou et al., 2002)
		+++	Mouse	spermatids	(Jensen, 2014)
	ESR2	++	Human	IM Germ cell specific	(Fietz et al., 2016; Fietz et al., 2014; Gibson and Saunders, 2012; Mäkinen et al., 2001; Saunders et al., 2002; Saunders et al., 2001; Saunders et al., 2003)
		+	Human	mRNA sperm	(Lambard et al., 2004)
		−	Human	IM	(Pelletier and El-Alfy, 2000)
		−	Rat	IM	(Gur and Aktas, 2020; Pilutin et al., 2014)
		+/+++	Rat	IM	(Menad et al., 2017; Oliveira et al., 2003; Turner et al., 2001; van Pelt et al., 1999)
		+/−	Rat	mRNA	(Kumar et al., 2018; Mowa and Iwanaga, 2001)
		++	Mouse	IM	(Bilinska et al., 2003; Cooke et al., 2017; Huang et al., 2016; Jensen, 2014; Zhou et al., 2002)
Leydig cells	AR	+++/+	Human	IM	(Iwamura et al., 1994; Kimura et al., 1993; Pelletier, 2000)
		+/−	Rat	IM	(Suarez-Quian et al., 1996)
		++/+	Rat	IM	(Figueiredo et al., 2016; O'Hara and Smith, 2015; Oliveira et al., 2003; Pelletier, 2000; Pilutin et al., 2014; Takeda et al., 1990; Tan et al., 2005; Turner et al., 2001; Vornberger et al., 1994; Williams et al., 2001a; Zhu et al., 2000)
		+++/++	Mouse	IM	(De Gendt et al., 2004; Krutskikh et al., 2011; Schauwaers et al., 2007; Smith et al., 2015; Welsh et al., 2009; Welsh et al., 2010; Zhou et al., 2002; Zhou et al., 1996)
	ESR1	−	Human	IM	(Fietz et al., 2016; Fietz et al., 2014; Mäkinen et al., 2001; Saunders et al., 2001; Saunders et al., 2003)
		−	Human	mRNA	(Fietz et al., 2016; Fietz et al., 2014)
		+	Human	IM	(Pelletier and El-Alfy, 2000)
		+++	Rat	IM	(Bremner et al., 1994; Fisher et al., 1997; Gur and Aktas, 2020; Lucas et al., 2008; Menad et al., 2017; Mizuno et al., 2011; Oliveira et al., 2003; Pilutin et al., 2014)
		++	Rat	mRNA	(Mowa and Iwanaga, 2001)
		+++	Mouse	IM	(Bilinska et al., 2003; Cooke et al., 2017; Zhou et al., 2002)
		−	Mouse	IM	(Jensen, 2014)
	ESR2	−	Human	IM	(Mäkinen et al., 2001; Saunders et al., 2002)
		+	Human	IM	(Gibson and Saunders, 2012; Saunders et al., 2003)
		+	Rat	IM	(Bilinska et al., 2003; Gur and Aktas, 2020; Menad et al., 2017; Pilutin et al., 2014)

Tissue	Receptor	Presence[1]	Species	Type of Study[1]	References
		−	Rat	IM	(van Pelt et al., 1999)
		+/−	Rat	IM	(Mowa and Iwanaga, 2001)
		+++/−	Mouse	IM	(Bilinska et al., 2003; Cooke et al., 2017; Jensen, 2014; Zhou et al., 2002)
Peritubular myoid	AR	+++	Human	IM	(Kimura et al., 1993; Pelletier, 2000)
		−	Human	IM	(Iwamura et al., 1994)
		+++/+	Rat	IM	(Bremner et al., 1994; Figueiredo et al., 2016; O'Hara and Smith, 2015; Oliveira et al., 2003; Pelletier, 2000; Pilutin et al., 2014; Suarez-Quian et al., 1996; Takeda et al., 1990; Tan et al., 2005; Turner et al., 2001; Vornberger et al., 1994; Williams et al., 2001a; Zhu et al., 2000)
		+++	Mouse	IM	(De Gendt et al., 2004; Schauwaers et al., 2007; Smith et al., 2015; Welsh et al., 2009; Welsh et al., 2010; Zhou et al., 2002; Zhou et al., 1996)
	ESR1	−	Human	IM	(Fietz et al., 2014; Mäkinen et al., 2001; Pelletier and El-Alfy, 2000; Saunders et al., 2001; Saunders et al., 2003)
		++	Rat	IM	(Gur and Aktas, 2020; Lucas et al., 2008; Oliveira et al., 2003)
		+++	Mouse	IM	(Cooke et al., 2017; Zhou et al., 2002)
		−	Mouse	IM	(Bilinska et al., 2003)
	ESR2	−	Human	IM	(Fietz et al., 2014; Mäkinen et al., 2001)
		+/++	Human	IM	(Saunders et al., 2002; Saunders et al., 2003)
		+/−	Rat	IM	(Gur and Aktas, 2020; Oliveira et al., 2003; Pilutin et al., 2014; van Pelt et al., 1999)
		+/−	Mouse	IM	(Cooke et al., 2017; Zhou et al., 2002)
Rete testis	AR	++	Human	IM	(Iwamura et al., 1994)
		++	Rat	IM	(Figueiredo et al., 2016; Williams et al., 2001a)
	ESR1	−	Human	IM	(Pelletier and El-Alfy, 2000)
		++/+	Rat	IM Epithelium ++/+; stroma +/−	(Fisher et al., 1997; Hess et al., 1997b)
	ESR2	+	Human	IM	(Pelletier and El-Alfy, 2000)
Efferent ducts	AR	−/+	Human	IM Epithelium proximal − ; near epididymis +	(Ungefroren et al., 1997)
		+++	Rat	IM Epithelium +++; smooth muscle +++	(Oliveira et al., 2004; Rivas et al., 2002; Williams et al., 2001a; Williams et al., 2000)
		+++	Mouse	IM	(O'Hara et al., 2011; Zhou et al., 2002)
	ESR	+++	Mouse	Receptor binding	(Schleicher et al., 1984)
	ESR1	+++/−	Human	IM Epithelium V nonciliated +++, ciliated cells −	(Rago et al., 2018; Saunders et al., 2001; Sullivan et al., 2019)
		+++/+	Human	IM Epithelium nonciliated +++, ciliated +	(Ergun et al., 1997)
		+++	Human	mRNA	(Légaré and Sullivan, 2020)
		+++	Rat	IM Epithelium +++; smooth muscle ++/−	(Atanassova et al., 2001; Chen et al., 1998; Fisher et al., 1997; Hess et al., 2011; Hess et al., 1997b; Oliveira et al., 2004; Oliveira et al., 2003)

Tissue	Receptor	Presence[1]	Species	Type of Study[1]	References
		+++	Rat	mRNA Epithelium +++; stroma +/−	(Mowa and Iwanaga, 2001)
		+++	Mouse	IM Epithelium +++; stroma −	(Arao et al., 2012; Cho et al., 2003; Cooke et al., 2017; Hoshii et al., 2007; Joseph et al., 2011; Lee et al., 2008; O'Hara et al., 2011; Zhou et al., 2002)
		+++	Mouse	mRNA	(Yao et al., 2017)
	ESR2	+++/−	Human	IM Epithelium +++; stroma +/−	(Pelletier and El-Alfy, 2000; Rago et al., 2018; Saunders et al., 2001)
		+++	Rat	IM Epithelium +++; smooth muscle ++	(Oliveira et al., 2004; Oliveira et al., 2003)
		−	Rat	IM Epithelium −; stroma −	(Yamashita, 2004)
		−	Rat	IM Epithelium −; stroma −	(Mowa and Iwanaga, 2001)
		+++	Mouse	IM Epithelium +++; stroma −/+	(Arao et al., 2012; Cho et al., 2003; Cooke et al., 2017; Lee et al., 2008; Zhou et al., 2002)
		−	Mouse	IM Epithelium −; stroma −	(Yamashita, 2004)
Epididymis	AR	+++	Human	IM Epithelium caput, corpus +++; cauda +	(Pelletier, 2000; Ungefroren et al., 1997)
		+++	Rat	IM Epithelium +++; stroma −/+	(Menad et al., 2014; Takeda et al., 1990; Williams et al., 2001a; You and Sar, 1998; Zhu et al., 2000)
		+++	Mouse	IM Epithelium +++; stroma +	(O'Hara et al., 2011; O'Hara and Smith, 2016; Zhou et al., 2002)
	ESR	++	Human	Receptor binding	(Murphy et al., 1980)
		++	Mouse	Receptor binding: apical, basal, clear cells	(Schleicher et al., 1984)
	ESR1	++	Human	mRNA caput ++; cauda +	(Légaré and Sullivan, 2020)
		−/+	Human	IM Epithelium	(Saunders et al., 2001)
		−	Human	IM Epithelium	(Ergun et al., 1997; Sullivan et al., 2019)
		+	Human	IM Epithelium +; stroma	(Rago et al., 2018)
		+	Human	IM Epithelium caput +; cauda −	(Kolasa et al., 2003)
		++/−	Rat	IM Epithelium caput + +/−; cauda +/−; stroma −	(Fisher et al., 1997; Gur and Aktas, 2020; Hess et al., 1997b; Kolasa et al., 2003; Menad et al., 2014; Sar and Welsch, 2000; Williams et al., 2000)
		−	Rat	IM Epithelium −; stroma −	(Atanassova et al., 2005 a; Atanassova et al., 2001; Yamashita, 2004)
		++	Rat	mRNA Caput	(Shayu et al., 2007)
		+	Rat	mRNA whole epididymis	(Jefferson et al., 2000)
		+	Mouse	mRNA Epithelium+; stroma +	(Mowa and Iwanaga, 2001)
		+/+++/−	Mouse	IM Epithelium Region specific principal cell + +/−; apical, basal cells, clear cells +++; stroma −	(Cooke et al., 2017; Hess et al., 2011; Hoshii et al., 2007; Jensen, 2014; Joseph et al., 2011; O'Hara et al., 2011; Yamashita, 2004; Zhou et al., 2002)

Tissue	Receptor	Presence[1]	Species	Type of Study[1]	References
	ESR2	+++	Human	IM Epithelium +++; stroma ++	(Saunders et al., 2001)
		+	Human	IM Epithelium caput +; cauda −; stroma −	(Kolasa et al., 2003; Pelletier and El-Alfy, 2000)
		++	Rat	IM Epithelium ++; stroma +/−	(Menad et al., 2014; Williams et al., 2000)
		+/−	Rat	IM Epithelium +/−; stroma −	(Gur and Aktas, 2020; Yamashita, 2004)
		−	Rat	IM Epithelium caput −; cauda −; stroma +	(Kolasa et al., 2003)
		−	Rat	mRNA Epithelium −; stroma +	(Mowa and Iwanaga, 2001)
		++	Rat	mRNA caput	(Shayu et al., 2007)
		+++	Mouse	IM Epithelium +++; stroma −	(Cooke et al., 2017; Jensen, 2014; Zhou et al., 2002)
		+/−	Mouse	IM Epithelium +/−; stroma −	(Yamashita, 2004)
		−/+	Mouse	mRNA	(Jefferson et al., 2000)
Vas deferens	AR	+++	Rat	IM Epithelium +++; stroma +/−	(Atanassova et al., 2005 a; Yamashita, 2004)
		++	Mouse	IM	(Yamashita, 2004; Zhou et al., 2002)
	ESR1	−	Rat	IM Epithelium −; stroma −/+	(Atanassova et al., 2005 a; Atanassova et al., 2001; Hess et al., 1997b; Williams et al., 2000; Yamashita, 2004)
		−	Rat	mRNA Epithelium −; stroma ++	(Mowa and Iwanaga, 2001)
		−	Mouse	IM Epithelium −; stroma +++/−	(Yamashita, 2004; Zhou et al., 2002)
	ESR2	++/−	Human	IM Epithelium	(Scobie et al., 2002)
		+	Rat	IM Epithelium +; stroma +/−	(Yamashita, 2004)
		−	Rat	mRNA Epithelium −; stroma +	(Mowa and Iwanaga, 2001)
		−/+	Mouse	mRNA	(Jefferson et al., 2000)
		+/−	Mouse	IM Epithelium +/−; stroma +/−	(Yamashita, 2004; Zhou et al., 2002)

[1]Estimate of expression. Immunohistochemistry (IM) unless otherwise indicated; mRNA can be isolated RNA or *insitu* hybridization; Receptor binding can be autoradiography or biochemical assay binding; PND, postnatal day; mo, months

Table 5.

Male reproductive phenotypes in gene targeted and transgenic animal models for the estrogen receptor pathways

Model	Name	Description	Key Phenotype	References
Esr1 (ERα)-null mouse	αERKO ERαKO Ex3 αERKO	Inactivation or deletion of *Esr1*	• Infertility; mating behavior disruption; increased blood T; +/− increased LH • Testis wt is normal at birth, followed by a transient increase and finally total atrophy by 185 days of age; dilation of seminiferous tubules PND 10–60, followed by atrophy; increased germ cell apoptosis; progressive spermatogenesis degeneration; sperm flagellar coiling in lumen; transplanted spermatogonia produce normal sperm; increased Leydig cell volume • Rete testis is overgrown with progression to extreme dilation PND 10–60 • Efferent ductule luminal dilation PND 6-adult; dysmorphogenesis; decreased epithelial height; loss of fluid reabsorption; decreased number of motile cilia and decreased expression of *Slc9a3, Slc9a3, Slc4a4,* AQP1,9, *Car2, Car14, Aqp4 and Umod*; increased expression of *Cftr, Slc26a3*; increased number of blind- ending tubules; glycogen granules present in proximal epithelium • Epididymis has abnormal narrow and clear cells; abnormal growth of epithelium in efferent ducts; sperm granuloma; decreased SLC9A3, CAR14 and SLC4A4; decreased cauda sperm count and sperm motility; decreased luminal osmolality; increased luminal pH; increased acrosome− reacted sperm; sperm flagellar angulation and coiling • Fetal diethylstilbestrol (DES) treatment has no effect on T	(Antonson et al., 2012; Couse et al., 2000; Delbes et al., 2005; Delbes et al., 2004; Dupont et al., 2000; Eddy et al., 1996; Gould et al., 2007; Goulding et al., 2010; Hess et al., 1997a; Hess et al., 2000; Hess and Carnes, 2004; Joseph et al., 2010a; Joseph et al., 2010b; Lee et al., 2001; Lee et al., 2000; Lee et al., 2009; Lubahn et al., 1993; Mahato et al., 2001; Nakai et al., 2001; Ruz et al., 2006; Shayu et al., 2007; Strauss et al., 2009; Toda et al., 2008; Toda et al., 2001; Weiss et al., 2008)
Esr1 (ERα)-null rat	Ex3αERKO	Global deletion of *Esr1*	• Infertility; decreased testis wt; dilation of seminiferous tubules • Efferent ducts not examined • Epididymis has traces of sperm	(Rumi et al., 2014)
Esr1 (ERα)-conditional deletion	ACTB-ERaKO	β-actin-Cre	• Infertility; increased T; decreased testis wt • Epididymis has decreased cauda sperm number	(Chen et al., 2009)
Esr1 (ERα)-conditional deletion	fERαKO	Fibroblast-specific protein (FSP)-Cre	• Fertile; testis is normal • Epididymis is normal	(Chen et al., 2012a; Chen et al., 2012b; Vitkus et al., 2013)
Esr1 (ERα)-conditional deletion	SmERαKO	Tgln (SM22α smooth muscle)-Cre	• Fertile; testis is normal	(Vitkus et al., 2013)

Model	Name	Description	Key Phenotype	References
			• Epididymis is normal	
Esrl/2 (ERα/ERβ)-null	Ex3ERαβKO DERKO	Double deletion of *Esrl* + *Esr2*	• Infertility; dilation of seminiferous tubules; increased T secretion; • Decreased cauda epididymal sperm number; decreased sperm motility	(Couse et al., 1999; Delbes et al., 2005; Dupont et al., 2000)
Esr2 (ERβ)-null	βERKO Ex3βERKO	Global deletion of *Esr2*	• Fertile; testis is normal • Epididymis is normal; normal sperm motility • Fertile; Testis appears normal at birth and adult; normal wt; +/− increased Leydig cell numbers; decreased Leydig cell volume; increased stimulated T secretion; increased LH; increased *Star, Cyp11a1, Hsd3b1, Cyp17a1, Hsdl 7b3* • PND2 increased number of gonocytes; increased gonocyte proliferation; decreased gonocyte apoptosis • Epididymis and sperm motility	(Antal et al., 2008; Couse et al., 1999; Delbes et al., 2004; Dupont et al., 2000; Gould et al., 2007; Imamov et al., 2004; Krege et al., 1998; Prins et al., 2001a; Prins et al., 2001b; Risbridger et al., 2001; Strauss et al., 2009; Weihua et al., 2001)
ESR1 overexpression	ESR1+	Transgenic ESR1	• Fertile; testis is normal; normal T	(Heath et al., 2011; Tomic et al., 2007)
Esr1 LBD mutant	ENERKI	Transgenic knock-in; agonist PPT activates	• Subfertile; rescued by PPT; increased T and LH • Testis normal to 12 wks; degeneration with aging; • Decreased sperm counts after 20 wks; normal efferent ducts	(Sinkevicius et al., 2008; Sinkevicius et al., 2009)
DBD mutant	NERKI, aERKO (−/AA; AA), KIKO, $ERα^{AA/–}$	DBD mutation on ERα-null background; precludes direct binding to ERE	• Maybe nonclassical ESR1; however, DNA binding changed from ERE to HRE • Fertile; normal testis wt; normal T, LH and FSH; occasional dilated seminiferous tubule; abnormal testis with aging • Delayed, diminished or reversed ESR1KO male effects; partial decrease SLC9A3, delay in decreased *Aqp1; Aqp9* normal • Normal sperm count	(Hewitt et al., 2014; Jakacka et al., 2002; McDevitt et al., 2008; McDevitt et al., 2007; Weiss et al., 2008)
DBD mutant	EAAE	ERα DBD mutation	• Completely inhibited binding to ERE and HRE motifs • Infertile; phenotype similar to ESR1KO • Reproductive tract not examined, but likely similar to ESR1KO	(Ahlbory-Dieker et al., 2009; Hewitt et al., 2014)
AF-1 mutant	ERα $AF\text{-}1^0$	Deletion of AF-1 (ligandindependent)	• Infertile; male reproductive tract not examined, but likely similar to ESR1KO	(Abot et al., 2013; Billon-Gales et al., 2009)

Model	Name	Description	Key Phenotype	References
AF-2 mutant	ERα AF-2^0	Deletion of AF-2 (LBD)	• Infertile; male reproductive tract not examined, but likely similar to ESR1KO	(Billon-Gales et al., 2011)
AF-2 mutant	AF2ER (KI/KI)	AF-2 Mutation	• ICI182,780 and tamoxifen (TAM) agonists through AF-1 • Infertile; seminiferous tubule dilation, slightly delayed from ESR1KO; increased T but not LH • Dilated rete testis and efferent ductules; decreased SLC9A3 and AQP9; decreased *Slc9a3, Aqp9, Car2*, and *Aqp1*; effects reversed by TAM	(Arao et al., 2012)
Esr1 D-domain mutant	H2NES	*Esr1* D-domain Hinge 2 mutation with nuclear export signal	• Cytoplasmic ESR1 only (nuclear ESR1 absent); However, possible post-translational problems • Infertile; similar to ESR1KO; testicular atrophy • Efferent ductules not examined, but likely similar to ESR1KO	(Burns et al., 2011; Stefkovich et al., 2017)
Nuclear-only ESR1	NOER	Membrane ESR1 localization lacking	• Retains functional nuclear ESR1; palmitoylation site (cysteine 451) ESR1 mutant • Infertile adults, but subfertile juvenile males; increased T • Testis wt increased; dilated seminiferous tubules and tubular atrophy; decreased DSP; coiled sperm tails • Dilation of rete testis and efferent ducts; abnormal epithelium similar to ESR1KO • Cauda epididymal sperm abnormalities; decreased sperm motility	(Nanjappa et al., 2016; Pedram et al., 2014)
Membrane-only ESR1	MOER	Transgene *Esr1* LBD	• Transgene containing multiple palmitoylation sites on an *Esr1KO* background • Infertile; male reproductive tract not examined, but likely similar to *Esr1KO*	(Pedram et al., 2009)
Aromatase-null	ArKO +/− soy free Cyp 19KO	Global deletion *Cyp19*	• Decreased fertility due to impaired mounting behavior • Normal spermatogenesis, with aging effects on testis • Normal efferent ducts; normal expressions of *Esr1, Esr2* and *Slc9a3* • Mild hypospadias	(Cripps et al., 2019; Robertson et al., 1999; Robertson et al., 2002; Robertson et al., 2001; Toda et al., 2008; Toda et al., 2001)
Aromatase over-expression	Int-5/ aromatase; AROM+	Transgenic male overexpression	• Subfertile to infertile; testis wt decreased (AROM+), 50% increased wt (Int-5/aromatase) • Leydig cell hyperplasia/hypertrophy and tumors; abnormal spermatogenesis; decreased serum T, increased E2; increased ESR1;	(Fowler et al., 2000; Li and Rahman, 2008; Li et al., 2006)

Model	Name	Description	Key Phenotype	References
			increased cyclin D1; increased testicular macrophage activation	
GPER1-null	GPERKO	Deletion of GPER1	• Fertile; no male reproductive phenotype; male reproductive tract not examined	(Otto et al., 2009; Prossnitz and Hathaway, 2015)
Estrogen sulfotransferase-null	ESTKO	Global deletion	• Fertile; testicular effects with aging; Leydig cell hypertrophic and hyperplastic • Decreased sperm motility with aging	(Qian et al., 2001)
Caput AR-null	CEARKO	Conditional deletion of caput epididymal epithelium AR	• Infertile; increased testis wt; fluid retention; dilation of rete testis and seminiferous tubules, followed by atrophy; progressive spermatogenesis degeneration; unilateral orchitis • Failure to develop initial segment epididymis; ESR1 expressed throughout caput epididymal epithelium; decreased epithelial height; disorganized smooth muscle layer; luminal occlusion with stagnant sperm; occasional inflammation in efferent ductules	(Krutskikh et al., 2011; O'Hara et al., 2011)
E2f4f/f;E2f5 +/− efferent ducts	E2f4f/f;E2f5 +/−;Vil-cre	Conditional deletion of efferent duct E2f4/5 transcription factors	• Infertile; dilation of rete testis and seminiferous tubules; fluid retention in testis; near total loss of cauda epididymal sperm • Decreased expressions of ESR1, AQP1 and CLU in efferent ductules; failure to develop motile ciliated cells	(Danielian et al., 2016)
LGR4-null and mutant	LGR4KO	Global deletion of LGR4/GPR48	• Infertile; testis fluid retention; dilation of rete testis and seminiferous tubules followed by atrophy; progressive spermatogenesis degeneration after onset of puberty • Efferent ducts and epididymis and were hypoplastic; proximal efferent ducts dilated with stagnant sperm; distal ducts occluded with sperm granulomas and inflammation; decreased proliferation; decreased epithelial heights; decreased expressions of ESR1, AQP1, SLC9A3 and Na/K-ATPase α1 in efferent ducts • Developmental defects: dilation of efferent ductules began on PND3; increased thickness of stroma in efferent ducts and epididymis; less convolution and elongation of the reproductive tract	(Hoshii et al., 2007; Li et al., 2010; Mendive et al., 2006)

[1]Abbreviations: wt, weight; mo, months; ESR1, estrogen receptor 1; ESR2, estrogen receptor 2; AR, androgen receptor; T, testosterone; FSH, follicle stimulating hormone; 4,4',4′-(4-Propyl-[1H] pyrazole- 1,3,5-triyl), PPT; SLC9a3, sodium/hydrogen exchanger 3; AQP, aquaporin; CAR2 and 14, carbonic anhydrase 2 and 14; SLC4A4, sodium bicarbonate cotransporter; CFTR, cystic fibrosis transmembrane conductance regulator; GPER, G protein-coupled estrogen receptor 1; *Cyp19*, aromatase; LBD, ligand binding domain; DBD, DNA binding domain; HRE, hormone response element, ERE, estrogen response element; ARE, androgen response element; AF-1 and -2, activation functions 1 and 2 domains; DSP, daily sperm production; PND, postnatal day

Table 6.

Comparison of *Esr1*KO and Neonatal Estrogen Treatment Models[1]

	***Esr1* KO**	**Anti-estrogen ICI**	**High DES**	**[2] High DES + T**	**High E2**	**Low DES + GnRHa or Flutamide**	**GnRHa**	**Flutamide**
AR	–	–	↓	–	↓	↓	–	sl↓
ESR1	↓	↓	-	-	-/↓		-	-
T	↑	--	↓	↑	↓		↓	↑
AQP1	↓	↓	↓		↓		-	
AQP9	↓	↓	↓	-	sl↓		↓	↓
Rete testis dilation	↑	↑	↑	-	↑	↑	-	-
ED dilation	↑	↑	↑	↑	↑	↑	-	-
References[3]	*1*	*2*	*3*	*4*	*5*	*6*	*7*	*8*

[1] Abbreviations: -, no effect; ↓, decreased effect; ↑, increased effect; sl, slight effect; AR, androgen receptor; ESR1, estrogen receptor 1; T, testosterone; E2, estradiol; AQP1,9, aquaporin; ED, efferent ductule; KO, knockout mouse; ICI, antiestrogen ICI 182,780; DES, diethylstilbestrol; GnRH, gonadotrophin-releasing hormone antagonist

[2] Testosterone treatment increases serum T, but also increases dihydrotestosterone (DHT) and E2, due to metabolic conversions (Amory et al., 2008; Robaire and Hamzeh, 2011). The increase in E2 would be nominal compared to the high dose of DES.

[3] References:

1: *Esr1*KO: (Arao et al., 2012; Eddy et al., 1996; Goulding et al., 2010; Hess et al., 1997a; Hess et al., 2000; Lee et al., 2000; Lee et al., 2009; Ruz et al., 2006; Zhou et al., 2001)

2: ICI 182,780: (Cho et al., 2003; Lee et al., 2000; Oliveira et al., 2005; Oliveira et al., 2001; Oliveira et al., 2003; Oliveira et al., 2002)

3. High dose DES: (Atanassova et al., 2005b; Fisher et al., 2002; Fisher et al., 1999; McKinnell et al., 2001; Pastor-Soler et al., 2010; Rivas et al., 2002; Sharpe et al., 1998; Sharpe et al., 2003)

4. High dose DES + T: (Atanassova et al., 2005b; Pastor-Soler et al., 2010; Rivas et al., 2003)

5. High dose E2: effect may depend on age (Fisher et al., 2002; Fisher et al., 1999; Naito et al., 2014; Pastor-Soler et al., 2010)

6. Low dose DES + GnRHa or Flutamide: (Rivas et al., 2002)

7. GnRH antagonist: (Fisher et al., 2002; McKinnell et al., 2001; Pastor-Soler et al., 2010; Rivas et al., 2002; Sharpe et al., 2003)

8. Flutamide: (Atanassova et al., 2005b; Fisher et al., 2002; McKinnell et al., 2001; Rivas et al., 2002)