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ESTROGENS IN MALE PHYSIOLOGY

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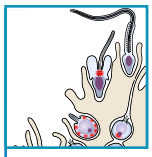


Cooke PS, Nanjappa MK, Ko C, Prins GS, Hess RA. Estrogens in Male Physiology. *Physiol Rev* 97: 995–1043, 2017. Published May 24, 2017; doi:10.1152/physrev.00018.2016.—Estrogens have historically been associated with female reproduction, but work over the last two decades established that estrogens and their main nuclear receptors (ESR1 and ESR2) and G protein-coupled estrogen receptor (GPER) also regulate male reproductive and nonreproductive organs. 17β-Estradiol (E2) is measureable in blood of men and males of other species, but in rete testis fluids, E2 reaches concentrations normally found only in females and in some species nanomolar concentrations of estrone sulfate are found in semen. Aromatase, which converts androgens to estrogens, is expressed in Leydig cells, seminiferous epithelium, and other male organs. Early studies showed E2 binding in numerous male tissues, and ESR1 and ESR2 each show unique distributions and actions in males. Exogenous estrogen treatment produced male reproductive pathologies in laboratory animals and men, especially during development, and studies with transgenic mice with compromised estrogen signaling demonstrated an E2 role in normal male physiology. Efferent ductules and epididymal functions are dependent on estrogen signaling through ESR1, whose loss impaired ion transport and water reabsorption, resulting in abnormal sperm. Loss of ESR1 or aromatase also produces effects on nonreproductive targets such as brain, adipose, skeletal muscle, bone, cardiovascular, and immune tissues. Expression of GPER is extensive in male tracts, suggesting a possible role for E2 signaling through this receptor in male reproduction. Recent evidence also indicates that membrane ESR1 has critical roles in male reproduction. Thus estrogens are important physiological regulators in males, and future studies may reveal additional roles for estrogen signaling in various target tissues.

I. HISTORICAL PERSPECTIVES ON ESTROGEN FUNCTIONS IN MALES

17β-Estradiol (E2) and other estrogens regulate many aspects of female reproductive development and function. Although estrogens were first detected in stallions in the 1930s (769), by the 1960s and 1970s, it became clear that males produced significant quantities of estrogens and men and males of other species had measureable circulating E2 concentrations. Furthermore, estrogen receptors (ER) were present in males during development and adulthood, and exposure to exogenous estrogens, especially developmentally, had deleterious effects on the male reproductive tract. Despite these data, roles for estrogen signaling in the normal male were difficult to determine for years, due both to a lack of good experimental systems to address this question and a paucity of clear end points for estrogen action. Over the last two decades, work using transgenic mouse models revealed that estrogens are critical for normal development and function of male reproductive and nonreproductive organs. This review traces the discovery of estrogen effects in males and provides an overview of current understanding of physiological roles for estrogens with an emphasis on more recent work with transgenic mouse models that have uncovered the complexity, breadth, and importance of estrogen actions in male reproductive tissues, as well as other organs.

Rapid research progress in the latter 20th century that elucidated E2 roles in female reproduction relied heavily on simple and powerful in vivo model systems. Hormonal fluctuations during the female estrous/menstrual cycle make it problematic to study E2 actions in intact animals. This was addressed partly by utilization of ovariectomized rodents (209, 210, 505). Ovariectomy and hormone replacement allows study of hormone actions in controlled and manipulable endocrine environments. In addition, these studies led to identification of E2-regulated biochemical, histological, and functional end points in female reproductive organs, and these robust end points facilitated E2 research.

This approach is illustrated by work of Finn and Martin (210), who described key E2 effects in ovariectomized rodents that shaped present understanding of E2 action in females.

Ovaries are the major source of circulating estrogens in females, but in males, testes produce only ~20% of circulating estrogens, with the remainder from local production by adipose, brain, skin, and bone, which convert testosterone (T) to estrogen through aromatase actions (708). Diffuse estrogen production in males meant that there was no simple method of producing estrogen-deficient states comparable to ovariectomized females. This hindered progress in this area. Despite E2 and ER presence in males, and known deleterious effects of perinatal estrogen treatment, there was no definitive evidence that E2/ER signaling was important in normal male reproduction. Similarly, it was unclear whether E2/ER signaling was involved in etiology or progression of naturally occurring male reproductive pathologies. All these factors constrained scientific interest and limited progress in this field.

Effects of estrogen administration on males both during development and adulthood were described before identification of ER or measurement of circulating estrogens in males. Early work showed that estrogens affected male behavior (201, 407). In addition, estrogen treatment altered development/function of the testis, prostate, and seminal vesicles (13, 59, 84, 356, 413, 445, 461, 465, 527). Estrogen effects on growth (413) and nonreproductive targets such as bone (312) and plasma proteins (475) were also described in males, as well as alterations in circulating luteinizing hormone (LH) and T concentrations (217, 671). Finally, early estrogen administration increased male susceptibility to carcinogen-induced liver cancer (730). Thus males responded to E2, but the question of whether E2 was important for normal development and function of reproductive and nonreproductive organs was not answered until development of various knockout mice decades later.

II. ESTROGEN PRODUCTION AND ACTIONS IN MALES

A. Estrogen Sources and Estrogen Concentrations

Although estrogens in males were first reported in the 1930s, when high estrogen concentrations were detected in stallion urine (769), accurate quantitation of estrogens in serum and other fluids was impossible until development of radioimmunoassay methodologies in the 1960s (750). These studies revealed low but measureable blood concentrations of E2 and other estrogens in various species of males, although circulating E2 concentrations in males exceeded those in ovariectomized female rats or rats in diestrus **(TABLE 1)**. In men, peripheral blood T concentrations of ~20 nM (495) are at least two orders of magnitude greater than E2 concentrations (30–200 pM; **TABLE 1**). In boars and stallions, conjugated estrogens such as estrone sulfate are uniquely elevated in both blood and semen, reaching nanomolar concentrations seen for androgens. Elevated E2 concentrations are found in rete testis fluid and in semen of many species **(TABLE 1)**. These vary with age, with higher concentrations prepubertally and age-related declines due to natural reductions in T, a E2 precursor (137).

In males, E2 production requires aromatase (*Cyp19a1*), a ubiquitous NADPH cytochrome P450 reductase enzyme (117). The testis was known to be involved in estrogen synthesis for years (769), but early studies focused on various T metabolites (49, 186, 533, 591). Despite descriptions of E2 binding in both testis and epididymis (161–163), well into the 1990s E2 was not considered a major regulator of male reproduction, at least in adults (reviewed in Ref. 292), and estrogen binding activity was considered a remnant of developmental processes influenced by estrogen action (260, 261, 456).

Initial work suggested that FSH-stimulated Sertoli cells are primary sources of estrogen in immature males, while LH-stimulated Leydig cells are the primary source in adult testis, as they express more aromatase than adult Sertoli cells (113, 275, 377, 385, 405, 501, 502, 533, 591, 700). However, in 1993, aromatase expression in adult testicular germ cells was first reported (496). Aromatase was localized in Golgi of round spermatids and throughout the cytoplasm of elongating and late spermatids **(FIGURE 1)**. Confirmed by Western and Northern analysis, aromatase activity in germ cells was comparable to that in Leydig cells (115, 328, 329, 496). In testis, the proximal promoter II regulates aromatase transcription, but numerous transcription factors drive this expression in a cell-specific manner, with Sertoli and germ cells showing specificity differing from Leydig cells (275).

Aromatase is expressed in male germ cells of several species **(TABLE 2)**, including mouse, rat, brown bear, bank voles, rooster, and human (reviewed in Refs. 115, 116). Aromatase is located in cytoplasmic droplets of sperm tails **(FIGURE 1)**, but becomes less intense as sperm traverse the epididymis (330). Carreau's laboratory reported that germ cells contribute ~62% of total testicular aromatase (405). Only a few species (boar, ram, and stallion) have germ cells that are not aromatase-positive (31, 285, 288, 643). It is unclear whether this reflects differences in aromatase antibodies or simply lack of aromatase in some species. Others report aromatase in epididymal epithelium and interstitium (111, 285, 545, 630), which could supply estrogen when sperm are not its primary luminal source. Thus, in humans and most experimental species, testicular germ cells and epididymal sperm serve as unique estrogen sources, which

Table 1. *Estrogen concentrations in males*

Source	Concentration	Species	Reference Nos.
Peripheral blood	3.6–91 pg/ml	Human	98, 112, 181, 207, 523, 762
	29–197 pM		125, 397, 495, 607
	43–464 pM (estrone)		397, 607
	40–145 pg/ml	Monkey	716
	2–175 pg/ml	Rat	58, 147, 168, 184, 340
	~70 pM	Mouse	77
	73.4 pg/ml	Horse	623
	64–250 ng/ml (estrone sulfate)		138, 571
	9–180 pg/ml	Bull	192, 232
	6.3 pg/ml	Ram	459
	~180 pg/ml	Boar	139, 624
	0.18 nM (total estrogens)		
	21.5 nM (estrone sulfate)		
	22.1–24.7 pg/ml	Avian	401
Testicular vein	104–200 pg/ml	Monkey	716
	19.0 pg/ml	Rat	168
	450 ng/ml (estrone sulfate)	Horse	623
	1.09 nM (total estrogens), 52.4 nM (estrone sulfate)	Boar	624
Testicular lymph	900 ng /ml (estrone sulfate)	Horse	623
Testicular homogenate	5–20 ng/g	Man	112
	39–751 pg/g	Rat	137, 340
	~4,500 pg/g (breeding season), ~100 pg/g (nonbreeding)	Avian	401
Rete testis	14–195 pg/ml	Monkey	716
	249 pg/ml	Rat	225
	11.5 pg/ml	Bull	232
	0.38 nM (total estrogens), 8.60 nM (estrone sulfate)	Boar	624
Semen	162 pg/ml	Man	98
	50–73 pg/ml (E2)	Horse	138, 399
	0.73–6.3 ng/ml (estrone sulfate)		138, 399, 571
	50–890 pg/ml	Bull	192, 232, 246
	430 pg/ml (E2), 860 pg/ml (estrone)	Boar	139

Many of these references, especially before 2010, used immunoassays to measure estrogen concentrations. It is now recommended that liquid chromatography, tandem mass spectroscopy be used when assaying for steroid hormones present in low concentrations. Unless otherwise indicated, measurements are for E2.

may target abundant ESR1 in efferent ductule and epididymal epithelium (296) **(TABLE 3)**.

Estrogens are inactivated through sulfoconjugation, which is catalyzed by estrogen sulfotransferase (EST) that is abundantly expressed in liver and other organs (657), and thus EST can affect estrogen concentrations in male organs. In males, highest EST concentrations and activities are in testis, but it also occurs in epididymis and ductus deferens (227, 302, 429, 584, 683). In testes, it is exclusively in Leydig cells, but in the mouse it is found in epithelia of the epididymis and ductus deferens, as well as in smooth muscle of the ductus deferens (683). However, its expression in prostate or seminal vesicle expression is controversial.

By inactivating estrogens, this enzyme regulates not only local estrogen exposure but also eventual biological effects. Epididymal epithelial EST (227, 304, 400, 477, 584, 683) may protect from excess estrogen (429) arriving through the efferent ducts as a result of CYP19A1 in spermatozoa (115, 496). Within the epididymal lumen, EST may stabilize acrosomal membranes through sulfation of membrane cholesterol (227, 584).

Testicular and epididymal EST is regulated by LH and androgens (683). Differential EST expression may contribute to differences in estrogen sensitivity among different strains of laboratory animals. For example, CD-1 mouse testes have the highest organ-specific activity (658) and are 16-fold less E2-sensitive, with 3.5 times more EST, than B6 mice (660). Testes from EST knockout mice showed Leydig cell hyperplasia and hypertrophy, with decreased testicular and epididymal weights (568). Sperm motility and fertility were also reduced. Expression of EST decreases with age,

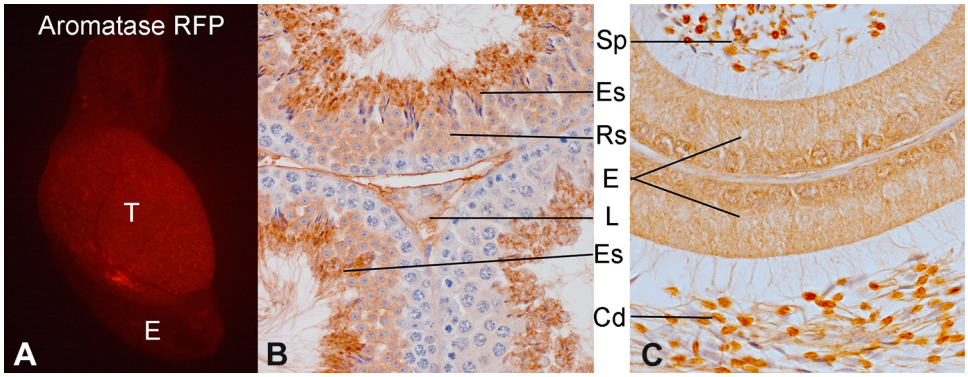


FIGURE 1. Aromatase (Cyp19) expression in male mouse reproductive tract. *A*: testis (T) and epididymis (E) from an adult (71-day-old) *Cyp19*RFP mouse showing RFP expression that is extensive within the testis, but lower in epididymis. *B*: adult testis showing immunohistochemical localization of aromatase in Leydig cells (L), round spermatids (Rs), and elongated spermatids (Es). *C*: adult caput epididymis showing immunohistochemical localization of aromatase in the cytoplasmic droplet (Cd) of sperm (Sp) in the tubular lumen. E, epithelium.

which may, along with age-related decreases in T production, contribute to increased serum E2 and decreased T/E2 ratios in elderly men.

B. ER Expression in Male Reproductive Organs

In the 1960s, studies by Jack Gorski, Elwood Jensen, Bert O'Malley, and others determined the mechanism of steroid hormone signaling (252, 453, 656). Early ER studies focused on female organs, but ER were demonstrated in males as well. In males, many studies examined brain regions such as hypothalamus (663). Later, ERs were reported in male reproductive as well as nonreproductive (liver, muscle, and kidney) organs (187, 410, 462). To characterize ER distribution, studies used either biochemical approaches (136) or the nascent technique of steroid autoradiography (663), which employed binding of radioactive estrogen tracers to

Table 2. *Aromatase presence in adult male reproductive tract tissues*

Species	Tissues	Reference Nos.
Mouse	Whole testis, Leydig cell, immature germ cell, spermatozoa	67, 121, 247, 330, 496, 724
Rat	Whole testis, Leydig cell, immature germ cell, spermatozoa, epididymal epithelium	34a, 78, 85, 86, 110, 238, 239, 282, 328, 329, 377, 388, 404, 405, 528, 533, 545, 551, 591, 630, 681, 686, 687, 688, 692, 699, 734, 735, 736, 744, 760
Dog	Leydig cell, Sertoli cell, immature germ cell	546, 733
Monkey	Immature germ cell, Leydig cell, immature germ cell	545, 692
Human	Immature germ cell, spermatozoa, epithelium of efferent ductule, epithelium of proximal epididymis	22, 24, 54, 93, 94, 111, 114, 117, 323, 382, 383, 384, 572, 600, 639, 692
Bird	Leydig cell, immature germ cell, spermatozoa	226, 378, 698
Fish	Total testis analysis, Leydig cell, immature germ cell	Dogfish (*S. acanthias*) (60, 156), European sea bass (*Dicentrarchus labrax*) (75, 160, 250), rainbow trout (*Oncorhynchus mykiss*) (358), Nile tilapia (*Oreochromis niloticus*) (357), sea bream (*Acanthopagrus schlegelii*) (396)
Amphibian	Total testis analysis	376, 510
Turtle	Total testis analysis	245, 552
Bear	Leydig cell, Sertoli cell, immature germ cell	318, 511, 690
Deer	Leydig cell	277
Boar	Leydig cell	145, 146, 149, 150, 224, 467, 732
Bull	Total testis analysis	704
Ram	Total testis analysis, Leydig cell	570, 615, 704
Stallion	Leydig cell, Sertoli cell, immature germ cell, epididymis	14, 193, 282, 288, 399, 400, 643
Bat	Leydig cell, Sertoli cell, germ cells	50
Squirrel	Leydig cell, Sertoli cell, germ cells	412, 567
Bank vole	Leydig cell, Sertoli cell, immature germ cell	68, 223, 362, 616

Table 3. *Localization of ESR1 in the male reproductive tract: species comparison*

Organ	Species																
	Bird	Fish	Amphioxus	Newt	Turtle	Lizard	Bat	Rat, Hamster, & Squirrel	Mouse & Vole	Dog	Cat	Goat & Ram	Marsupial	Horse	Boar	Monkey	Human
Testis	+	+	+	+	+	+	+	+/−	+	+	+	+/−	+	+	+	+/−	+/−
Leydig cell		+		+	+		+/−	+/−	+	+	+	−	+	+	+	+	+/−
Sertoli cell		−	+	+	−		+/−	+/−	+/−	−	+		+	+	+	−	+/−
Germ cell		−/+	+	+	−	+	+/−	+/−	+/−	−	−		+	+	+	+/−	+/−
Myoid cell*					−		−	+/−	+	+	+		−	+	−		+/−
Rete testis	+				+		+	+/−		+	+	−				−	+
Efferent ducts	+				+	+	+	+	+	+	+	+				+	+
Epididymis	+				+	+	+	+/−	+	−	+					+	−
Vas deferens								+/−	+/−	−	+						+
Reference Nos.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17

Most references show data for ESR1 using immunohistochemistry or Western blotting; however, in some cases, mRNA presence was determined by RT-PCR, ribonuclease protection assay, or in situ hybridization. For some tissue, data from different laboratories may be inconsistent. Positive data are indicated by + and negative results by -. Data variation likely depends on quality of tissue preservation, antibody specificity, and variations in laboratory techniques. In cases where there are no data, the square is blank. *Peritubular myoid cell. Discussions for numerous species are available in previous reviews (115, 295, 296). Reference numbers refer to the following: 1. Bird: (327, 401, 513). 2. Fish: black porgy, *Acanthopagrus schlegeli Bleeker*, protandrous hermaphrodite fish (279); channel catfish (746); rainbow trout, *Oncorhynchus mykis* (83); teleost fish, *Sparus aurata* (654); killifish, mummichog, *Fundulus heteroclitus* (262); European eel, *Anguilla anguilla* (468); Asian swamp eel, M*onopterus albus* (183). 3. Amphioxus, *Branchiostoma belcheri* (199). 4. Newt, *Triturus marmoratus marmoratus* (26). 5. Turtle, *Trachemys scripta* (158, 245). 6. Lizard, Italian wall lizard, *Podarcis sicul* (707). 7. Bat (50, 520, 521). 8. Rat, hamster, and squirrel: (33, 78, 137, 190, 211, 219, 297, 359, 370-372, 412, 424, 425, 460, 472, 516, 518, 542, 543, 551, 599, 605, 608, 636, 677, 718, 751, 759, 760). 9. Mouse and vole: (12, 68, 77, 154, 260, 311, 314, 333, 414, 436, 494, 632, 673, 751, 763). 10. Dog: (492, 618, 676). 11. Cat: (492, 617). 12. Goat, ram: (256-258, 441, 606). 13. Marsupial, tammar wallaby, *Macropus eugenii*: (102). 14. Horse: (236, 282, 530, 535). 15. Boar: (398, 476, 573). 16. Monkey: (90, 211, 280, 342, 542, 609, 610). 17. Human: (23, 58, 88, 123, 177, 196, 202, 203, 205, 249, 270, 359, 384, 435, 439, 525, 542, 543, 610, 622, 637).

identify ER. Steroid autoradiography was used for years to visualize ER expression, especially in developing organs. Steroid autoradiography was supplanted by immunohistochemistry in the 1980s, and recent data regarding ER distribution in male organs comes primarily from immunohistochemistry. These studies have benefited from constantly improving antibodies as well as methodological advances (e.g., antigen retrieval) that facilitated ER immunohistochemistry. In addition, methodologies continue to be developed, such as the new mouse model that expresses red fluorescent protein (RFP) under the control of ESR1 and ESR2 promoters that we describe here.

Early autoradiographic studies assumed that E2 binding resulted from a single ER (252, 335, 504, 656). As immunohistochemistry for localizing ER gained preeminence in the 1980s, it was initially assumed that ER immunostaining in reproductive tissues (65, 361) was equivalent to autoradiographic ER localization (663). Indeed, agreement between immunohistochemical and autoradiographic ER localization when both techniques were used simultaneously in organs such as uterus (65) supported this idea. However, identification of a second ER (371) indicated that autoradiographic and immunohistochemical data differed. The new ER, now known as estrogen receptor 2 (ESR2) or estrogen receptor beta (ERβ) to differentiate it from the original ER [now known as estrogen receptor 1 (ESR1) or estrogen receptor alpha], was originally identified in rat prostate, but had wide distribution in reproductive and nonreproductive organs (371).

Identification of ESR2 indicated that autoradiographic E2 binding resulted from both ESR1 and ESR2 in target organs, while ESR1 immunostaining identified only ESR1, but presumably not ESR2 (depending on antibody specificity). Even this was oversimplified, as subsequent work revealed another ER, now known as G protein-coupled estrogen receptor (GPER) (204, 577, 678). This protein is associated with cell membranes and endoplasmic reticulum and binds estrogens such as E2, although with less affinity than ESR1/ESR2 (204, 577, 678). More recent studies have revealed that in addition to the 66 kDa ESR1, some cells express two truncated ESR1 isoforms, ERα36 and ERα46 (126, 216, 722). These variants are found both in the nuclear/cytoplasmic as well as membrane compartments. Some evidence indicates that these variant forms of ESR1 may be important in breast cancer (723), but their role in normal female or male physiology is totally unknown. Thus older steroid autoradiography showing ER expression represents binding activity of several molecules (ESR1, ESR2, GPER, ERα36, and ERα46) and must be interpreted carefully.

1. Expression of ER in adult males of various species

Expression of ESR1 **(FIGURE 2, TABLE 3)** and ESR2 **(FIGURE 2)** occurs throughout adult male mouse reproductive tracts,

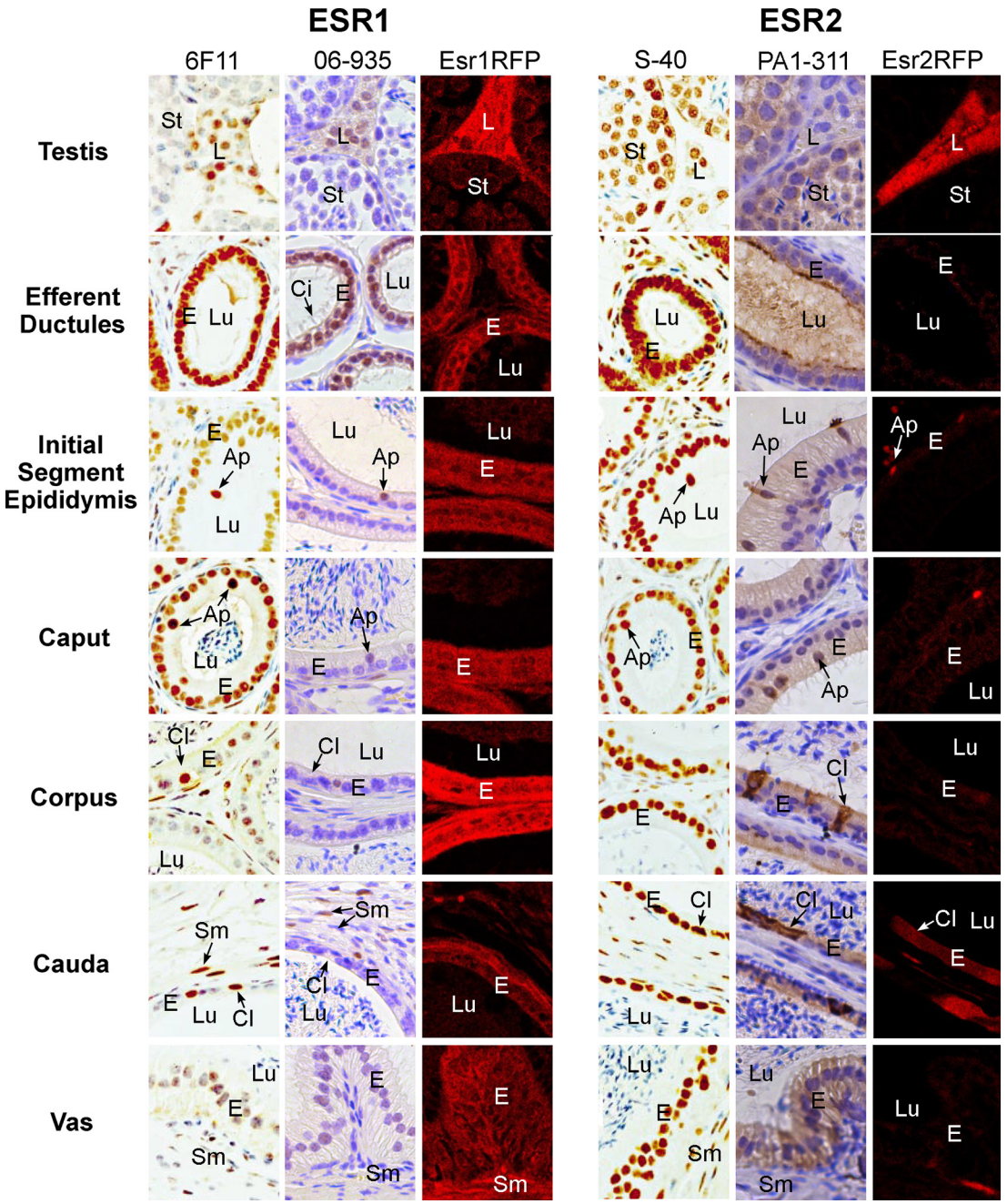


FIGURE 2. Expression of ESR1 and ESR2 in male mouse reproductive tract. Representative samples of immunohistochemical staining with 2 different ESR1 and ESR2 antibodies and red fluorescent protein (*Esr*RFP) mice. In *Esr1*RFP (63 days of age) and *Esr2*RFP mice (59 days of age), cell lineages expressing *Esr1* or *2* will subsequently show RFP and may not correlate with current immunohistochemical staining. ESR1 staining is primarily nuclear using 6F11 [NCL-ER-6F11 antibody (Novocastra, Newcastle upon Tyne, UK)] and labels many more epididymal cell types than does the anti-ESR1 antibody 06–935 (Millipore, NH). Testis shows ESR1 exclusively in interstitial or Leydig cells (L) with no immunostaining in seminiferous tubules (St), although low fluorescence was seen with RFP. Efferent ductules show strong epithelial nuclear ESR1 staining with both antibodies, but cytoplasmic staining was also seen, especially with 06–935. In epididymis, apical (Ap) and clear cells (Cl) show strong nuclear staining with 6F11, but staining differences were observed in other cell types with the two antibodies. ESR2 staining was more widespread than ESR1. However, the S-40 ESR2 antibody (Dr. Saunders, Univ. of Edinburgh) showed intense nuclear staining, while PA1–311 (Thermo, Waltham, MA) shows considerable or exclusive cytoplasmic staining. The lack of RFP fluorescence in efferent ductules and most epididymal regions may indicate that ESR2 expression in these regions is delayed past day 60. In rats, ESR1 is expressed in efferent ducts and epididymis earlier than ESR2 (605), although in humans the opposite occurs (627). In the pig epididymis, ESR2 does not appear until after puberty (109). E, epithelium; Lu, lumen; Sm, smooth muscle; Ci, cilia. [Images for ESR1 using the 6F11 antibody and for ESR2 using the S-40 antibody were modified from Zhou et al. (763).]

although expression patterns for each are unique (763). In testis, most antibodies localized ESR1 only in Leydig and peritubular cells **(FIGURE 2, TABLE 3)**. However, ESR1 mRNA and protein in Sertoli cells were reported (265, 414, 422). Conversely, ESR2 is found in Leydig, peritubular, germ, and Sertoli cells. One commonality has been efferent ductules **(FIGURES 2 AND 3)**, where ESR1 expression is threefold higher than uterus (297) and intense across species (296). Both ESR1 and ESR2 are expressed in nonciliated epithelial cells of all species and ciliated cells of most species **(FIGURE 3)**. ESR2 is also expressed in stroma. Epididymis expresses both ESR1 and ESR2, but as in other organs, expression patterns are unique and regionally variable. Both ESR1 and ESR2 occur in epithelium and stroma of ductus deferens, although again expression of the two receptors is not superimposable.

Although most studies utilized rodents or humans, ESR1 has been reported in various mammalian and nonmammalian vertebrates **(TABLE 3)**. In testis, ESR1 mRNA and immunostaining were not detected in some studies, but were strongly positive in others **(TABLE 3)**. Other studies suggested that ESR1 mRNA expression changes prepubertally (333). Epididymal studies were also inconsistent, with some studies showing no epididymal *Esr1* mRNA (211), while in mice, nearly all epithelial cells are positive (342, 763).

In contrast to ESR1, most reports suggest that ESR2 protein and mRNA are expressed ubiquitously in male reproductive organs of several species (101, 296, 492, 518, 563, 608, 610, 763), but varies with species, age, antibody, and organ. For example, mouse testicular *Esr2* mRNA was strongly expressed from postnatal day 1–5, then nearly absent from days 12–26. Mouse epididymis showed essentially no expression prepubertally (333). In rats, ESR1 is expressed much earlier than ESR2 in efferent ducts and epididymis (605), and some studies report no epithelial expression (359). However, in humans, the opposite was found, with *Esr1* mRNA present first (627). In pigs, epididymal *Esr2* mRNA does not appear until postpubertally (109). Thus species differences and developmental expression patterns must be considered.

While commonalities were noted in terms of ESR1/ESR2 expression in primates versus mice, there were also differences. For example, although ESR2 was expressed throughout primate testis, as in mice, ESR1 expression was minimal in primate testis. Similar to mice, ESR2 was expressed in stroma and epithelium of human efferent ductules, ductus deferens, and epididymis. Conversely, ESR1 was abundant only in nonciliated cells of efferent ductules, but minimal in epididymal epithelium, despite pronounced staining in some mouse epididymal epithelial cell types. Similar variations in other species have resulted in significant controversy **(TABLE 3)**, with differences in antibodies, species, fixation, and animal ages all contributing.

The *Esr*RFP mice provide a new method for identification of *Esr1* and *Esr2* expression in males, with RFP being localized in cytoplasm of cells expressing these receptors (101). This mouse can be used to compare RFP localization with immunohistochemical data **(FIGURE 2)**. This model allows visualization of ER expression in whole or even groups of reproductive organs. For example, *Esr1*RFP was more intense in epididymis than testis, the opposite of *Esr2*RFP **(FIGURES 2 AND 4)**. In mouse testis, this is consistent with the predominately interstitial immunohistochemical ESR1 expression **(FIGURE 2)**. However, staining with two different ESR1 antibodies differed in some epididymal areas, while efferent ductal epithelium stained intensely with both. Previous studies have shown higher *Esr1* mRNA in corpus than other epididymal regions (296), consistent with *Esr1*RFP data, but not immunostaining.

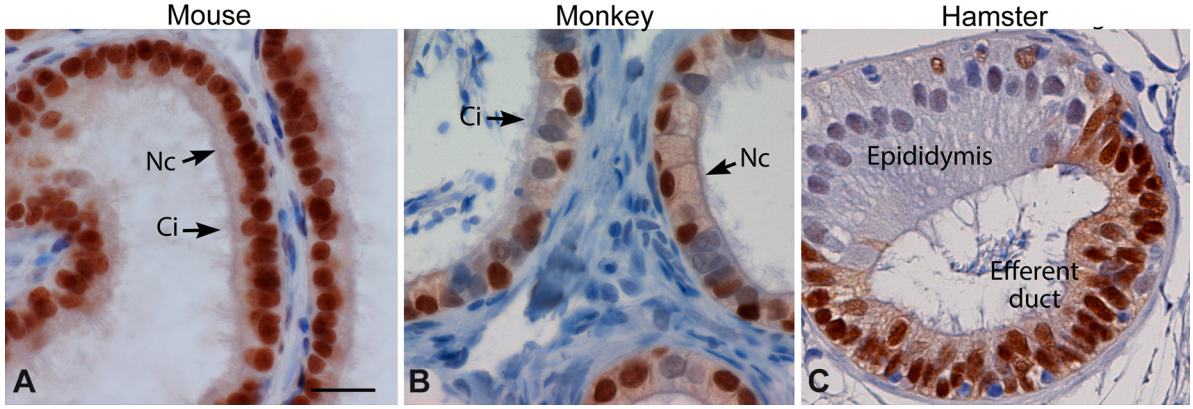


FIGURE 3. Efferent duct expression of ESR1 in 3 mammalian species. Efferent ductules from mouse (*A*), marmoset monkey (*B*), and hamster (*C*) show intense immunostaining for ESR1. In most species, both ciliated (Ci) and nonciliated (Nc) cells have strong reactions in the nucleus, with some light cytoplasmic staining. However, the monkey ciliated cells were inconsistent, with some staining slightly positive and others being negative. The hamster image shows the efferent duct/initial segment junction, with intense staining of efferent duct epithelium but minimal epididymal staining.

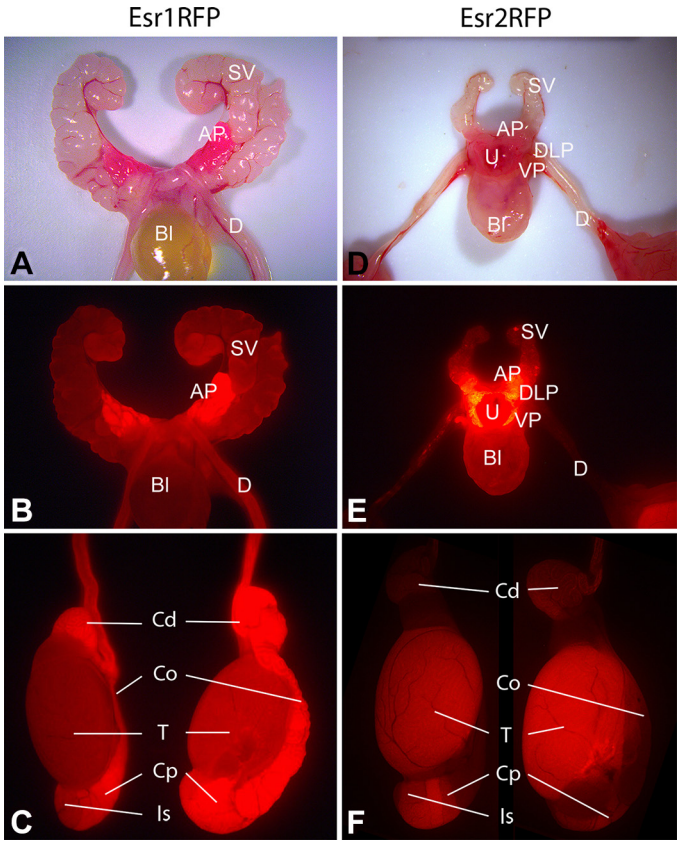


FIGURE 4. Expression of ESR1 and ESR2 in male mouse reproductive tract visualized using *Esr*RFP mice. In *Esr*1RFP (*A–C*) and *Esr*2RFP (*D–F*) mice, red fluorescent protein (RFP) expression is under the control of the respective steroid receptor. *A* and *B*: whole mounts of adult (63-day-old) *Esr*1RFP reproductive tract photographed with normal light (*A*) or by exposing the tissue to light at 549 nm and then looking at fluorescence emission at 574 nm using a Zeiss HBO 100 illuminating system (*B*). The anterior prostate (AP; also called the coagulating gland) showed strong ESR1 expression (*B*) compared with much weaker ESR1 expression in seminal vesicles (SV) and ductus deferens (D), while expression in the bladder (Bl) was at the limit of detection. *C*: in whole mounts of adult (63-day-old) *Esr*1RFP testis and associated structures, the testis (T) is lightly positive, but more intense fluorescence is seen in the initial segment (Is), caput (Cp), corpus (Co), and cauda (Cd) regions of the epididymis. In juvenile (22-day-old) *Esr*2RFP male mice (*D* and *E*), ESR2 showed intense expression in ventral and dorsolateral prostate (VP and DLP, respectively), while AP and urethra (U) showed modest expression, Bl and SV showed minimal expression, and the ductus deferens (D) was essentially negative. *F*: in adult (59-day-old) *Esr*2RFP male mice, the IS and Cp of the epididymis showed clear signal for ESR2, while ESR2 expression in the Co and Cd regions of the epididymis were basically undetectable. In contrast to the epididymis, where ESR1-RFP expression (*C*) was more dominant compared with ESR2-RFP (*F*), in the testis ESR2-RFP expression (*F*) was stronger than ESR1-RFP expression (*C*).

High ESR1 expression in anterior prostate **(FIGURE 4)** contrasts with much lower expression in neighboring seminal vesicles. Expression of ESR2 in both ventral and dorsolateral prostate is especially intense **(FIGURE 4)**, contrasting with lower expression in anterior prostate and urethra and minimal seminal vesicle expression.

One limitation of this model is the requirement that normal *Esr* transcriptional activity involves only one allele, as the other allele is null due to iCre insertion in place of an initiation codon of either *Esr*1 or *Esr*2 to drive the universal promoter of the RFP transgene (101). However, RFP will be expressed in a cell lineage beginning with initial *Esr* expression. Thus cells expressing *Esr*1 in early development but subsequently losing this expression still show RFP fluorescence, revealing past ESR1 expression even when ESR1 is no longer produced.

Studies have reported increased proliferation of Sertoli cells in response to E2 and weak environmental estrogens during development (55, 122, 237, 411, 425), with rapid estrogen responses in males possibly involving GPER present in Sertoli cells, germ cells, epididymis, and sperm (115, 132, 236, 296, 424). Furthermore, mouse and rat Sertoli cells were reportedly ESR1 negative (518, 551, 763) or positive (137, 414, 422, 425, 718). In **FIGURE 2**, Sertoli cells were negative with two different antibodies, although there was weak expression in *Esr1*RFP mice. However, lack of ESR2 fluorescence in *Esr*2RFP mice was surprising as ESR2 Sertoli cell immunolocalization has been consistent. This may reflect limitations of this model when expression is low. A Sertoli cell line (SK11) derived from young mice expressed ESR2 but not ESR1, and following transient transfection of a reporter gene with an estrogen response element, these cells showed a dose-response to 5-androstane-3-β,17β-diol, a 5α-reductase metabolite with high ESR2 affinity (652). Interactions of both ERs and GPER may help to explain estrogen actions on testicular cells as well as epididymis and sperm (748, 752, 753). Therefore, future studies are required to understand the presence and activity of the estrogen receptors in male reproductive tracts and the significance of different staining patterns and receptor interactions.

2. Developmental ER expression

Perinatal exposure of males to natural or synthetic estrogens such as diethylstilbestrol (DES) produces long-term changes in male reproductive organs in rodents and other species (reviewed in Ref. 455), suggesting functional ER are present in developing reproductive organs. Early autoradiographic studies showed that ER was present as early as fetal day 13 in mesenchyme of mouse urogenital sinus, the precursor of male prostate and bulbourethral glands, and on day 16 of gestation in mesenchyme of Wolffian ducts (148, 308, 664, 665), which form epididymis, ductus deferens, and seminal vesicles. Efferent ductules were the first male reproductive structure to show nuclear epithelial E2 binding during development (148, 308, 664, 665). These results suggested that fetal male reproductive organs are estrogen targets during their early ambisexual stage, consistent with literature showing early estrogen exposure produces male reproductive abnormalities (455). As organs such as prostate, bulbourethral gland, epididymis, ductus deferens, and

seminal vesicles differentiate, they maintain ER expression (148, 308, 614, 664). In addition, ER expression occurs in human fetal testis (110). Thus fetal male reproductive organs and their precursors are targets for endogenous and exogenous estrogens.

C. Deleterious Effects of Early Estrogen Administration in Humans and Animals

1. Developmental DES exposure in humans and animals

In the mid 1970s, McLachlan and colleagues (456, 489, 490) treated mid-gestation pregnant mice with DES, producing adult reproductive abnormalities in offspring, including cryptorchid testes, epididymal cysts, seminal vesicle abnormalities and increased infertility. The window for DES effects extends into postnatal life, and Bern, Iguchi, and colleagues (317) demonstrated that neonatal DES treatment also produced adult male reproductive abnormalities and infertility.

DES-induced developmental defects in efferent ducts and rete testis were similar to those in *Esr*1KO mice (32, 212, 213). These abnormalities were sometimes accompanied by reduced AR, and this, along with discovery that exogenous estrogens decrease ESR1 (516), illustrates the potential of early estrogen exposure to alter subsequent receptor exposure and the balance between androgen and estrogen signaling in males.

Animal DES studies were driven in part by pioneering studies by Herbst et al. in 1971 (283) showing that young women whose mothers had taken DES during pregnancy were prone to develop a rare cancer, vaginal clear cell carcinoma. At approximately the same time as McLachlan's animal studies were published, additional studies of male offspring of women given DES during pregnancy indicated that these men had higher incidences of reproductive problems (62, 63) that correlated well with animal models.

2. Environmental estrogens

Environmental estrogens (xenoestrogens) are a heterogeneous class of chemicals, both man-made and natural, with estrogen-mimicking activity. These compounds can be synthetic industrial pollutants or pesticides (e.g., bisphenol A or BPA, methoxychlor, kepone, DDT/DDE, atrazine), pharmaceuticals (e.g., DES, ethinyl estradiol), or naturally occurring phytoestrogens (e.g., genestein, daidzein). Mechanisms of xenoestrogen actions are varied and can include transactivation of nuclear ESR1 and ESR2 as well as activation of membrane-initiated signaling through membrane ESR1, ESR2, and/or GPER, triggering multiple downstream cascades (725). Consequently, xenoestrogen exposure can lead to variable responses between compounds and end points examined. For in-depth discussions of this topic, refer to recent reviews (251, 334, 454). It is notable that due to variable receptor affinities, selectivity for membrane versus nuclear receptors, activation of other steroid and thyroid receptors in addition to ERs, distinctive metabolism and compound-specific pharmacokinetics, xenoestrogens will not necessarily reproduce E2 effects but rather initiate distinct and often nonpredictable and nonmonotonic dose responses that differ between end organs (701). As a result, low doses of xenoestrogens can interfere with natural estrogen actions, even in the presence of higher circulating E2 concentrations.

III. TRANSGENIC ANIMAL MOUSE MODELS FOR STUDYING ESTROGEN PHYSIOLOGY IN MALES

A. Estrogen Receptor 1 Knockout Mice

Testosterone and its metabolite dihydrotestosterone (DHT) facilitate development and continuous release of spermatozoa and act on the epididymis to enable fertilization-competent sperm maturation and storage (501). However, spermatozoa must travel through a complicated region between the rete testis and caput epididymis, which includes the efferent ductules and initial segment of the epididymis (291). This region expresses androgen receptor (AR) but is also uniquely dependent on estrogen and ESR1, specifically in efferent ducts (299, 342), and DHT in the initial segment, where 5α-reductase activity predominates (582). These tubules require luminal delivery of these steroids, as circulating hormones do not fully maintain the epithelium following proximal efferent ductule ligation (200, 583). It is noteworthy that rat efferent duct epithelium expresses more ESR1 than any other male or female tissue (297), and efferent ductule fluid is rich in E2 due to collective activities of Cyp19a in sperm and different testicular cells (115, 330, 496).

Efferent ductules are a series of tubules connecting the rete testis and epididymis. Their epithelium consists of ciliated cells whose beat appears to mix luminal fluid and water-absorbing nonciliated cells whose morphology and physiology resemble kidney proximal tubules (142, 290, 291, 321). Efferent duct epithelium reabsorbs up to 96% of luminal fluid (140–142, 321) and concentrates sperm before their epididymal entry and is responsible in part, along with the caput epididymis, for maintaining an optimal sperm maturation microenvironment (341, 343). As in kidney, physiology of water movement in efferent ducts and epididymis is highly coupled to ion transport (142, 272, 320).

Expression of numerous genes and proteins is altered in male reproductive tracts of *Esr1*KO mice and rodents treated with ER-specific estrogens or anti-estrogens (248,

296, 341, 375, 544, 629, 754). However, only two physiological roles for estrogen have been demonstrated: ESR1 is required for *1*) fluid resorption by efferent ductule epithelium (293) and *2*) maintenance of sperm morphology and motility (343). Both of these depend on expression of various ion and water transport proteins (293, 514, 598, 761) to establish luminal environments that maintain optimal pH, osmolality, and sperm concentration.

The original *Esr1*KO mice had low-level expression of a truncated ESR1 (191, 254, 421), complicating interpretation of their reproductive phenotype. However, key aspects of the *Esr1*KO phenotype, such as fluid resorption impairment and secondary testicular effects, were replicated in exon 3 *Esr1*KO mice (21, 129, 189, 254), *Esr1*KO rats (597), EAAE mice in which *Esr1* DNA binding was blocked (6), AF2ERKI mice with mutated AF-2 (activation function domain) (25), and NOER (nuclear-only ESR1) mice (482). ESR1 inactivation also causes Leydig cell hypertrophy and elevated serum T, but these effects are indirect due to increased LH (12, 253) and increased Leydig cell T production capacity (9).

In *Esr1*KO mice, the major morphological effect was impaired efferent ductule epithelial differentiation, resulting in decreased epithelial height and loss of structures associated with fluid reabsorption in nonciliated cells **(TABLE 4)**. In addition, there were reductions in several proteins responsible for fluid/ion equilibrium (341–343, 598, 761). These changes caused a more than twofold luminal dilation in *Esr1*KO efferent ductules compared with WT **(TABLE 4, FIGURE 5)** with fluid accumulation in rete testes and seminiferous tubules (191, 253, 293, 294, 480, 682). Motile cilia numbers were also reduced **(TABLE 4, FIGURE 5)**, and those present showed an abnormal beat (294). These changes were observed as early as postnatal day 10 (394) and could have been due to abnormal development. Treatment of adult animals with a potent anti-estrogen, Faslodex (717), confirmed that ESR1 plays a major physiological role in efferent ducts **(FIGURE 5)**. Adult Faslodex treatment mimicked many

Table 4. *Key morphological effects of ESR1 disruption in male reproductive tracts*

Morphological Feature	Change
Testis	
Testis size	Transient increase
Seminiferous tubule lumen	Dilation
Seminiferous tubule	Atrophy with aging
Rete testis	Dilation, glycogen accumulation
Leydig cell	No effect
Sertoli cell	No effect
Germ cells	Decreased number but normal morphology
Efferent ducts	
Lumen	Dilation
Blind-ending tubules	Increased number
Epithelium	Decreased height
Nucleus	Decreased size
Nonciliated cell	Decreased volume
Microvilli	Decreased number and size
Endocytic apparatus	Decrease
Water channels	Decrease
SLC9A3	Decrease
CAII	Decrease
CFTR	Increase
Ciliated cell	Decrease cilia number, abnormal beat
Epididymis	
Initial segment	Abnormal growth and displacement of epithelium in efferent ducts
Apical cells	Abnormal
Clear cell	Abnormal
Luminal sperm	Decreased concentration
Sperm motility	Decrease
Sperm morphology	Abnormal

Morphological changes were noted in one or more of the following references: 191, 253, 292–295, 394, 480, 597, 598, 682, 731.

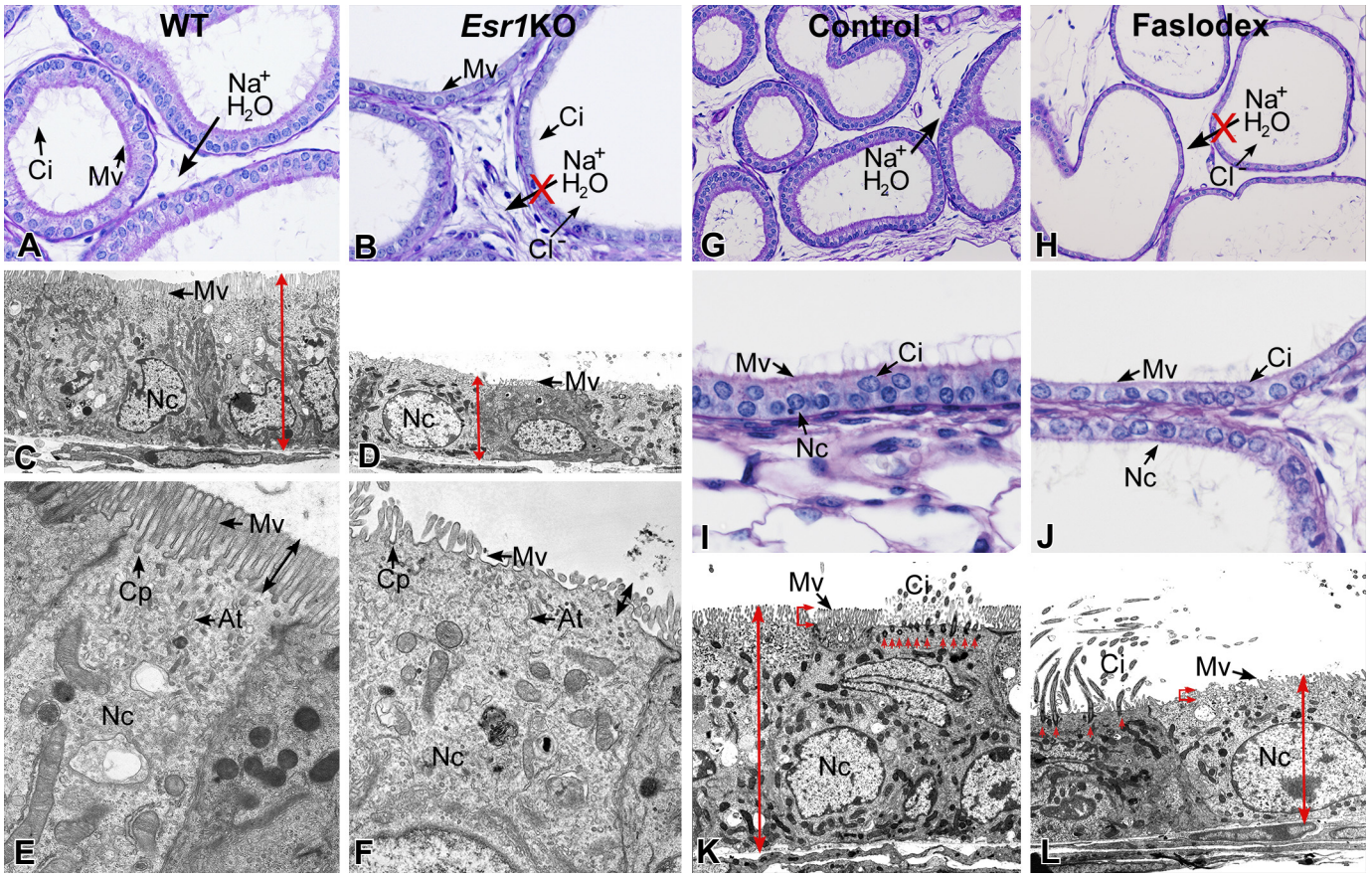


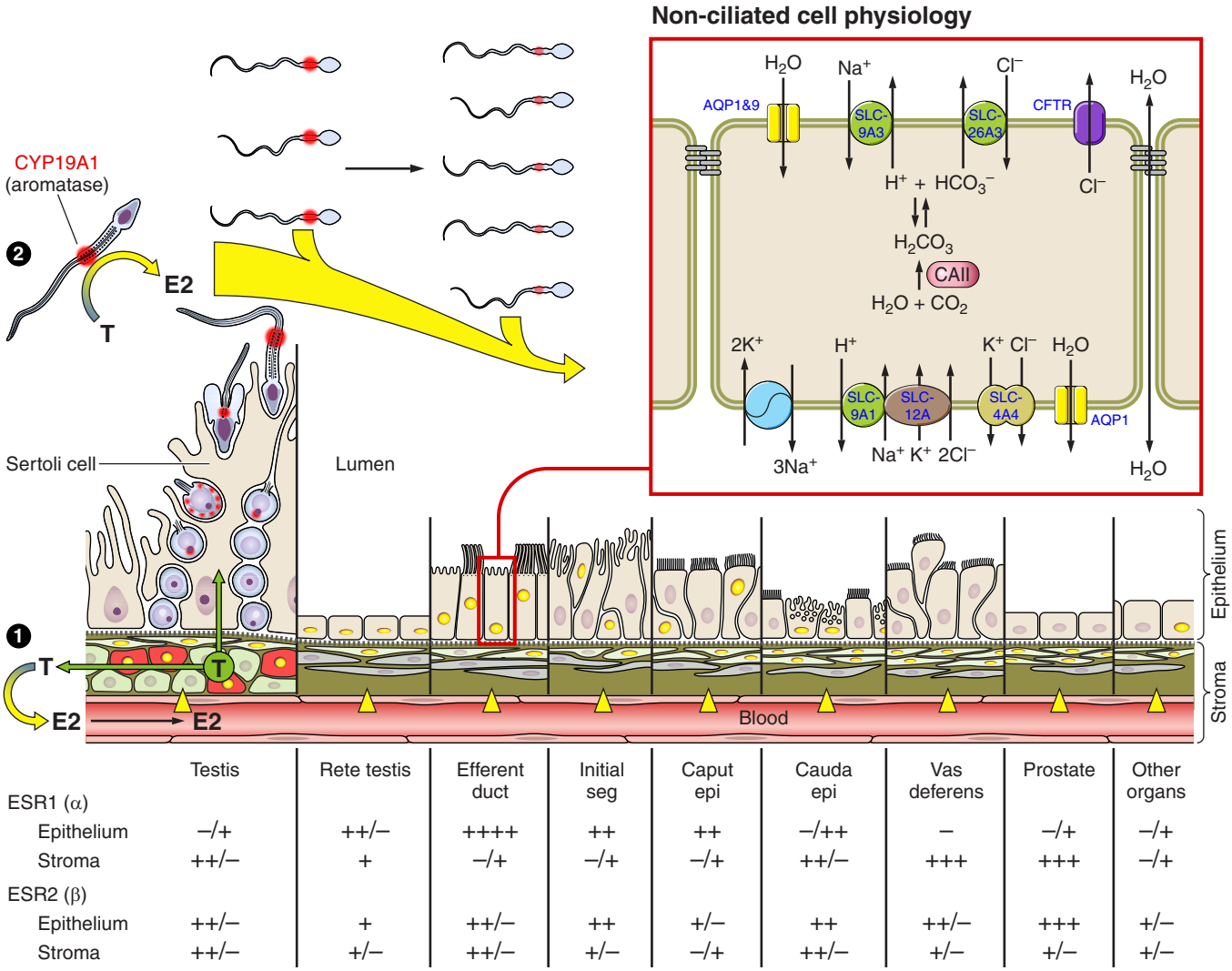
FIGURE 5. Efferent ductule morphology in *Esr1*KO and anti-estrogen (Faslodex)-treated mice. *A* and *B*: light microscopy of adult wild-type (WT) and *Esr1*KO efferent ductules. WT ducts have a periodic acid (PAS)-positive brush border of microvilli (Mv) on nonciliated cells, which move sodium ions (Na^+) and water (H_2O) to concentrate luminal sperm that are transported into the epididymis. Long cilia (Ci) project into the lumen. *Esr1*KO ducts have a dilated lumen and reduced epithelial height. Epithelium is deficient in microvilli, and cilia are fewer and shorter. Sodium transport and water resorption are inhibited, but chloride ion (Cl^-) secretion into the lumen is increased, adding to water accumulation. *C–F*: transmission electron microscopy of wild-type and *Esr1*KO efferent ductules. WT epithelium is taller than *Esr1*KO (double-headed red arrows). WT nonciliated cells (Nc) show a well-developed luminal border of microvilli (double-headed black arrows), coated pits (Cp), and apical resorption tubules (At). *Esr1*KO duct epithelium is short, and microvilli of nonciliated cells are short or absent and coated pits and apical tubules are reduced in apical cytoplasm. *G–J*: light microscopy of adult control and anti-estrogen (Faslodex)-treated efferent ductules. Control ducts have a smaller lumen but taller epithelium than Faslodex-treated mice. Sodium and water transport are actively moved into the interstitium but inhibited in treated epithelium. Nonciliated cells in controls have a PAS^+ brush border of microvilli and ciliated cells support long cilia projecting into the lumen (*I*), in contrast to Faslodex-treated epithelia (*J*). *K* and *L*: transmission electron microscopy of control Faslodex-treated efferent ductules. Control epithelium is tall (*K*) compared with Faslodex-treated ducts (*L*). Control nonciliated cells have a well-developed luminal microvillous border, while treated duct epithelium has short microvilli. Control ciliated cells have numerous basal bodies (red arrowheads) in the apical cytoplasm to support cilia projecting into the lumen, in contrast to reduced cilia in treated cells (*L*). [The *Esr1*KO and Faslodex-treated images from Hess et al. (298), with permission from Taylor & Francis Group, LLC; and from Hess (289), with permission from the Brazilian College of Animal Reproduction.]

*Esr1*KO phenotypes such as efferent duct and rete testis dilation, but only caused partial seminiferous tubular atrophy without increased testis weight (133, 393). Furthermore, there was a 50-day latency in the response to blocking ESR1 activity. Similar findings were also seen with rats (515, 519).

A model of estrogen production, receptor expression, and action is shown in **FIGURE 6**. This model also incorporates the primary E2 effects on efferent ductule epithelial physiology.

B. Estrogen Receptor 2 Knockout Mice

Initial studies of *Esr2* knockout (*Esr2*KO) mice did not observe the dramatic male or female reproductive changes seen in *Esr1*KO mice, and the male reproductive phenotype in double-*Esr1*/*Esr2*KO mice was similar to *Esr1*KO males, confirming that ESR1 is the functionally dominant ER in males (189, 368). In some species ESR2 shows considerably less expression compared with ESR1, also suggesting a limited role for ESR2. The original *Esr2*KO



	Testis	Rete testis	Efferent duct	Initial seg	Caput epi	Cauda epi	Vas deferens	Prostate	Other organs
ESR1 (α)									
Epithelium	–/+	++/–	++++	++	++	–/++	–	–/+	–/+
Stroma	++/–	+	–/+	–/+	–/+	++/–	+++	+++	–/+
ESR2 (β)									
Epithelium	++/–	+	++/–	++	+/–	++	++/–	+++	+/–
Stroma	++/–	+/–	++/–	+/–	–/+	++/–	+/–	+/–	+/–

FIGURE 6. Estrogen synthesis and its targets in male reproductive tract. This figure summarizes the variation reported for the localization of estrogen receptors (ESR) in epithelia and stroma of testis, rete testis, efferent ductules, initial segment (seg), caput, cauda epididymis (epi), vas deferens, prostate, and other organs. Only nuclear ESR1 (yellow color) are represented. However, in some tissues, cytoplasmic and membrane ESRs have been documented. Receptor localization varies widely between species and with various antibodies (305). In adult testis, CYP19A1 (red color), the cytochrome P450 aromatase enzyme responsible for converting T to E2, is principally found in spermatids and mature sperm in seminiferous tubules and Leydig cells. These two sources of estrogen in the male reproductive system are directed to separate physiological pathways: *1*) E2 from Leydig cells may target the seminiferous epithelium, although Sertoli and germ cells appear to be inconsistent in their ESR1 expression. This minor source of estrogen enters the blood and targets stromal and epithelial tissues not only in the reproductive tract but also all other ER-expression organs. *2*) Germ cell production of E2 begins within seminiferous epithelium and continues with the localization of aromatase in the cytoplasmic droplet of spermatozoa transported in the lumen of the reproductive tract. The major target of luminal E2 is efferent ductule epithelium, where ESR1 expression is the highest in the body. The major function of efferent ductules is reabsorption of nearly 90% of the luminal fluid, which increases sperm concentrations entering the initial segment. This major physiological function, under ESR1 regulation, involves kidney-like physiology of the nonciliated cells (outlined in the red box), of which several genes are directly *Esr1* regulated (296, 342).

was fertile, but these mice had increased Leydig cell numbers and decreased germ cells due to gonocyte or germ cell apoptosis (175, 253) and hyperplastic prostatic changes with aging (322, 727).

Analysis of these mice was complicated by the presence of alternatively spliced ESR2 transcripts (189, 368), similar to the truncated ESR1 in the original *Esr1*KO mice (421). Therefore, a true null *Esr2*KO was developed (19). Mating defects in these mice have been reported (19), although this is controversial, suggesting that lack of ESR2 does not impair sperm production/motility, but may impair mating. The use of selective ER inhibitors in vivo and in vitro, which bypass hypothalamus-pituitary-testicular

feedback loop problems (501), have shown that selective ESR2 agonists in rats impair fertility and spermiation, potentially by altering the tubulobulbar complex in seminiferous epithelium, without LH or FSH effects (188, 375). Overall, the data indicate some ESR2 effects on male reproduction.

C. Aromatase Knockout Mice

Aromatase knockout (*Cyp19*KO) mice, developed to test the hypothesis that estrogen is essential for many physiological systems, had normal efferent ducts and rete testis morphology and normal spermatogenesis until they began to age (339, 502, 585–587, 682). An aromatase inhibitor similarly has no effect on efferent ductules and fluid physiology (248, 534, 693). However, soy-free diets lacking phytoestrogens, which have high ESR2 affinity, accelerated spermatogenic declines in *Cyp19*KO mice (586), suggesting ESR2 involvement in spermatogenesis.

For years, E2 treatment was the primary approach for demonstrating estrogen roles in male reproduction (36, 271). However, E2 treatment creates interpretational problems due to alterations in hypothalamus-pituitary-testicular feedback (501). Furthermore, in some male tissues, ESR1 shows constitutive expression and possible activity in the absence of luminal estrogen in both *Cyp19*KO mice and following castration (516, 682). In testis, in vitro E2 treatment of seminiferous tubules increases ESR1 (697), but decreased efferent duct ESR1 in vivo (516), possibly explaining the absence of efferent ductal phenotypes in *Cyp19*KO mice (682). This suggests that efferent duct morphology and physiology, while ESR1 dependent, may not require direct E2 stimulation.

Other studies have shown that ESR1 may be activated in a ligand-independent manner (450, 503, 554, 642, 729) or non-E2 ESR1 ligands may be active in some male tissues (512, 550). Although *Cyp19*KO mice showed only a long-term role for direct estrogen actions in testis, other studies are uncovering roles in Sertoli and germ cells, including spermatozoa (124, 422–425; for reviews, see Refs. 115, 296, 342). Therefore, care must be taken when interpreting studies using *Cyp19*KO mice and estrogen treatment of males. One of the ESR1 target genes in efferent ducts is *Slc9A3* (341–343, 598, 761), which not only contains estrogen response elements (ERE), but also androgen response elements (ARE) in its promoter region (685). Thus the potential for dual regulation of efferent ductule physiology, involving an estrogen/androgen balance, would help to explain some of the complexities observed in the male. Future studies must also evaluate the status of ERs and their associated cofactors in a species- and tissue-specific manner.

D. *Gper* Knockout Mice

In addition to ESR1 and ESR2, GPER, a G protein-coupled receptor originally described as orphan receptor GPR30, also functions as an ER or cooperates in ER activation (204, 577, 678). Activation of GPER results in increased intracellular calcium and phosphatidylinositol 3-kinase (PI3K). Lack of GPER impairs E2 actions in cancer cells, but its effects on normal reproductive development and function are unclear. Several *Gper* knockouts (*Gper*KO) were developed, but no female reproductive abnormalities were reported (325, 442, 522, 719; for a review, see Ref. 566).

A variety of testicular cell types express GPER, including germ, peritubular, Leydig, and Sertoli cells (28, 58, 131, 203, 236, 296, 406, 444, 602). Expression of GPER has been reported in germ cells during various stages of spermatogenesis in rodents, and direct GPER-mediated effects on germ cells have been suggested (131, 645). Leydig cells express GPER and signaling through GPER has been implicated in both Sertoli cell proliferation and maturation and maintenance of fertility (423, 424, 753). Finally, GPER expression in peritubular cells has been linked to sexual maturation and maintenance of fertility (617). Signaling through GPER may also play a significant role in the epididymis and in expression of GPER in the epididymis (444, 544) and in posttesticular maturation of sperm (236).

Despite documented GPER expression/actions in the male tract, *Gper*KO males are fertile and without reproductive abnormalities, indicating that GPER is dispensable for male reproduction. However, *Gper*KO males are obese with insulin resistance and dyslipidemia (266, 628), phenotypes also seen in mice lacking ESR1 or aromatase. An absence of GPER has metabolic effects in males, as well as effects on cardiovascular, beta cell, and skeletal physiology (566). Therefore, GPER's overall role in E2 signaling in males remains unclear (235, 406, 431).

E. Transgenic Mice Lacking Membrane ESR1 Signaling

1. Development of mice lacking membrane ESR1

Although ESR1 is predominately cytoplasmic and nuclear, ~5% of this protein localizes to cell membranes (1, 541). This process requires ESR1 palmitoylation at cysteine-451 in mice (538), which was recently used to develop transgenic nuclear-only estrogen receptor 1 (NOER) mice where alanine was substituted for cysteine-451 (C451A) in mouse ESR1. Alanine cannot be palmitoylated, which precludes cell membrane localization of ESR1. Resulting mice expressed normal amounts of fully functional nuclear ESR1 (nESR1), but membrane ESR1 (mESR1) was essentially eliminated in both reproductive and non-reproductive tissues.

Development of NOER mice resulted in identification of critical mERS1 actions in females (3, 538) and suggested that mESR1 might also be important in males. We recently examined male reproductive development and function in transgenic NOER mice (482) and reported that mESR1 is essential for male fertility and that absence of mESR1 causes extensive deleterious male reproductive abnormalities. In adult NOER testes, rete testis (RT) was strikingly enlarged compared with WT and was comparable to that in *Esr1*KO males (191). Also paralleling *Esr1*KO males (191, 293), seminiferous tubule luminal diameters were increased in NOER mice, with increased degeneration in NOER seminiferous tubule epithelium.

Decreased sperm production and motility is a hallmark of *Esr1*KO mice. In 8-mo-old NOER mice, sperm production and motility were reduced by 85 and 60%, respectively, compared with WT. However, many caudal epididymal sperm in NOER mice remain viable, indicating that reduced motility did not simply reflect sperm death. Decreased NOER sperm motility was accompanied by structural abnormalities in over 95% of cauda epididymal sperm. Abnormalities included increases in folded or coiled midpieces and tails, as well as increased numbers of headless sperm.

A critical question for understanding effects of loss of mESR1 on cauda epididymal sperm is when these abnormalities arise. Do structural abnormalities in epididymal sperm originate during development, or following release into the seminiferous tubular lumen? We observed that NOER sperm morphological abnormalities were absent in seminiferous tubules containing late-stage VIII spermatids, when sperm are released into the lumen **(FIGURE 7*A*)**. Conversely, in the RT **(FIGURE 7*B*)**, extensive tail folding and coiling previously seen in epididymal sperm were observed. Thus NOER sperm abnormalities arise following release from seminiferous epithelium, likely due to altered fluid environments in the rete, efferent ductules and epididymis. These findings are again consistent with *Esr1*KO and antiestrogen-treated mice (133, 343, 482).

In NOER mice, luminal diameters in proximal efferent ductules (adjacent to RT) were increased ~50% in NOER mice, while proximal ductule epithelial height was reduced ~50% in NOER **(FIGURE 8)**. Previous work has shown that *Esr1*KO mice show similar changes in these parameters (290, 296).

Matings of homozygous adult NOER males (5- to 8 mo-old) with fertile WT females never yielded pregnancies. However, when juvenile NOER mice that were ~2 mo of age were placed with proven WT breeders, some of the NOER males sired litters, although litter size was reduced. Thus adult NOER males are infertile, but juvenile NOER males are transiently fertile during development.

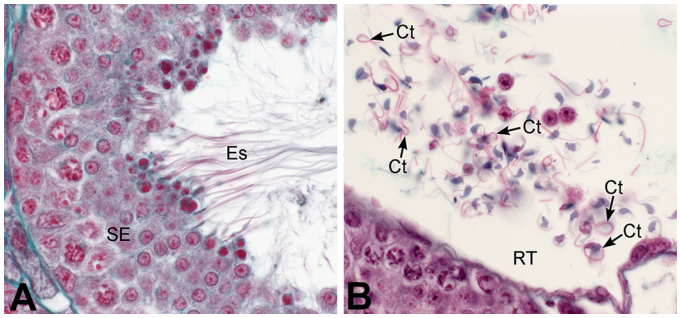


FIGURE 7. Structural abnormalities of NOER mouse sperm arise in the post-seminiferous tubular environment. Forty-day-old NOER male testes were fixed and stained with Masson's trichrome. *A*: seminiferous tubular epithelium at spermiation (stage VIII) shows normal sperm with straight tails. *B*: rete testis region of NOER mice shows high numbers of abnormal sperm with coiled tails (CT). SE, seminiferous epithelium; Es, elongated spermatids.

Loss of mESR1 in NOER males, even with continued nESR1 presence, leads to extensive reproductive changes culminating in severe structural and functional sperm abnormalities and eventually infertility. These findings identify a previously unknown role for mESR1 in normal E2 signaling in males and indicate that mESR1 expression is necessary for male fertility (482), as it is for female fertility (538).

2. Estrogen mediated effects through membrane ESR1

Despite attenuated E2-induced responses in NOER male and female mice, these mice do not totally lack E2 responsiveness, and establishing the mechanism of this effect is a critical goal. Membrane estrogen receptors activate PI3K and mitogen-activated protein kinase (MAPK) pathways, and have other actions. Protein kinase activation by E2/mESR1 signaling may be crucial for phosphorylation and recruitment of cofactors to nESR1 after E2 binding, regulation of ESR1 synthesis, and degradation and other effects that result in impaired E2 actions.

Extensive evidence indicates mESR1 may also be involved in epigenetic changes arising from early estrogen exposure, and this may be a critical effect mediated through membrane signaling. In the presence of E2, mESR1 interacts with the p85α regulatory subunit of PI3K, leading to activation of protein kinase B/AKT (153). DES or other estrogens can act through mESR1 to increase signaling through the PI3K/AKT pathway in neonatal rodent uteri (89, 259, 745). Critically, increased PI3K/AKT signaling then alters histone methylation. The most critical regulator of epigenetic changes such as histone methylation is the polycomb repressive complex 2 (PRC2) enzyme complex. The PRC2 is a histone methyltransferase that has major effects on gene function by silencing gene activity. The PRC2 functions by adding up to three methyl groups at lysine-27 of histone H3 (H3K27) to form trimethylated histone H3 (H3K27me3).

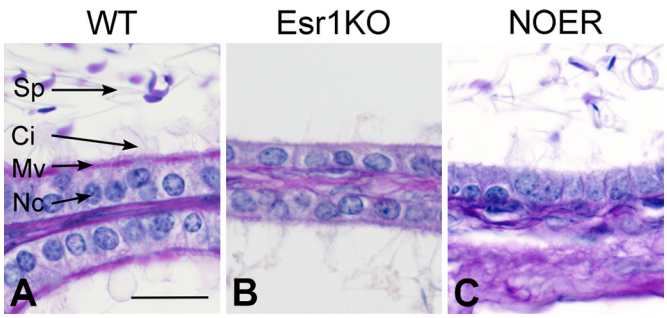


FIGURE 8. Efferent ductule epithelium from adult wild-type (WT), *Esr1*KO, and NOER (nuclear-only ESR1) mice. Periodic acid-Schiff (pink) and hematoxylin (blue) staining. Bar = 20 μm. *A*: WT epithelium is short columnar with ciliated (Ci) and nonciliated (Nc) cell. Nonciliated cells have a prominent brush border of microvilli (Mv) lining the lumen that contains diluted population of sperm (Sp). *B*: *Esr1*KO epithelium is shorter in height than WT, with significant loss of apical cytoplasm and much of the nonciliated microvillus border. Cilia numbers are reduced. *C*: NOER epithelium is shorter in height than WT, lacks a microvillus border, and shows reduced apical cytoplasm, similar to *Esr1*KO mice. Cilia also are reduced. Abnormal sperm with coiled tails are seen in the lumen.

Activity of PRC2 is regulated primarily by expression of enhancer of Zeste homolog 2 (EZH2), the catalytic subunit of the PRC2 complex that provides methyltransferase activity. In response to mESR1 signaling, activated AKT phosphorylates and inactivates EZH2, causing reduced H3K27me3. Since H3K27me3 is a repressive mark, this reduction leads to hyperresponsiveness to estrogen in adulthood (259), resulting in increased tumorigenesis and other reproductive diseases such as leiomyoma in adult rodents, and potentially in women, after early estrogen exposure (705, 757, 768). Estrogen effects mediated through EZH2/H3K27me3 appear to be a main mechanism of epigenetic estrogen effects (89, 259, 745), and EZH2 regulation of H3K27me3 may be involved in prostate cancer, emphasizing its potential role in E2 effects mediated through mESR1 in males (705).

IV. ABNORMALITIES IN ESTROGEN PRODUCTION OR ER EXPRESSION IN HUMANS

Progress in understanding the role of estrogen in men has been made in the past two decades through identification and characterization of human patients with mutations in ESR1 or aromatase. The first man lacking functional ESR1 was reported in 1994 (647) **(TABLE 5)**, although a woman (569) and three siblings (2 females and 1 male) (57) lacking ESR1 function were recently reported. Shortly thereafter, humans lacking aromatase were identified (176), with 13 reported cases of loss of function mutations in *CYP19A1* in men now known **(TABLE 5)**.

A. Estrogen Deficiency in Men

Estrogen deficiency due to loss-of-function mutations in *CYP19A1* [also known as aromatase deficiency (AD)] in men is characterized by normal male sexual differentiation and pubertal development (47, 82, 107, 130, 287, 386, 433, 434, 464, 469). However, AD cases with birth defects such as hypospadias (81) and cryptorchidism (81, 387, 433) are known. Pregnant mothers carrying male or female fetuses with homozygous aromatase mutations frequently show progressive virilization that resolves after parturition (173, 287, 464, 469). The extent of maternal virilization depends on the specific *CYP19A1* mutation, since fetuses with even 1% of normal aromatase activity do not trigger maternal virilization (264).

Postpubertally, AD men and women have sought medical attention for bone pain or continuous linear growth (47, 82, 107, 130, 287, 386, 433, 434, 464, 469). Serum hormone concentrations analysis revealed normal to elevated LH, follicle stimulating hormone (FSH), and T as well as undetectable E2. GnRH stimulation produced robust LH release and subnormal FSH release (287, 434). Similar gonadotropin and T changes were also reported in humans given aromatase inhibitors as young adults (447), further suggesting an E2 role in negative feedback of gonadotropic hormones in men.

Adult men with AD typically show normal testicular size, although macro-orchidism (469) or micro-orchidism (107, 433) have been reported. The role of E2 in human testis function, spermatogenesis, and fertility is unclear, since there were no consistent findings in testes histology or sperm analysis in AD men. Furthermore, due to patient noncompliance for further analysis as well as preexisting conditions such as cryptorochidism and hypospadias, conclusions regarding E2's role in testis function are difficult. Nonetheless, Sertoli cell-only seminiferous tubules (433), hypospermatogenesis (107, 434), seminiferous epithelial atrophy and degeneration and spermatogenic arrest (107) have been reported. No changes in Leydig cells morphology have been reported. Furthermore, semen analyses revealed a range from normal to severe oligospermia and normal to immotile sperm (107). In AD men, dietary exposure to phytoestrogens or other environmental estrogens may obscure effects of endogenous estrogen deficiency, since phytoestrogen exposure in *Cyp19*KO mice delays testicular degeneration (586). Fertility of these men is unknown, with the exception of one case (107); however, based on testicular and sperm abnormalities in AD patients, fertility in AD men may be impaired.

Almost all AD men are tall, with eunuchoid body proportions, open epiphyses, genu valgum, osteopenia, osteoporosis, younger than chronological bone age, bone pain, increased bone turnover, and frequent fractures **(TABLE 5)** as a result of decreased bone mass and mineral density. Administration of E2 to these patients induced epiphyseal closure, improved bone deposition, and alleviated bone pain **(TABLE 5)**. Similarly, epidemiological studies show men

Table 5. *Effects of loss of aromatase or ESR1 in men*

Original and Related Case Reports	Type of Mutation, Subject Age	Reproductive Effects	Skeletal Effects	Metabolic Effects
		Aromatase mutation		
Morishima et al. 1995 (469), Bilezikian et al. 1998 (66)	Single point mutation, 27 yr	Virilization of mother; normal sexual and pubertal development; elevated T, LH, and FSH; low E2 levels; macro-orchidism; no semen analysis; heterosexual orientation and behavior	Tall stature, osteopenia, osteoporosis, younger bone age (14 yr), low bone mass and mineral density, unfused epiphyses	Hyperinsulinism, normal glucose, dyslipidemia, BMI 32.5
Carani et al. 1997, 1999 (107, 108), Rochira et al. 2000 (588)	Single point mutation, 0.4% aromatase activity, 31 yr	Normal sexual and pubertal development, normal T, slightly elevated FSH, upper normal LH and undetectable E2, micro-orchidism, infertility, oligospermia with immotile spermatozoa. Hypospermatogenesis and germ cell arrest, heterosexual orientation and behavior	Tall stature and bilateral genu valgum, bone pain, open metacarpal and phalangeal epiphyses, and younger bone age (14.8 yr)	Normal insulin and glucose, dyslipidemia, BMI 27.6
Deladoey et al. 1999 (173)	Base pair deletion in *CYP19* gene causing truncated, inactive protein, infant	Normal sexual differentiation; maternal virilization; normal serum-free T and high androstenedione at birth, which decreased by 1 month; normal testes descent		
Herrmann et al. 2002, 2005 (286, 287)	Frameshift mutation resulting in premature stop codon and truncated aromatase protein, 27 yr	Maternal virilization, normal sexual and pubertal development, high T and FSH, normal LH, low E2, normal testicular volume, oligospermia, reduced sperm motility, normal sperm morphology and vitality, heterosexual orientation and behavior	Tall stature, low bone mass and mineral density, increased linear bone growth, genu valgum, kyphoscoliosis, and pectus carniatus	Normal plasma glucose and insulin dyslipidemia, BMI 30.9
Maffei et al. 2004 (433), Carrani et al. 2005 (106), Rochira et al. 2007 (590)	Point mutation resulting in truncated aromatase protein, 29 yr	History of bilateral cryptorchidism, normal sexual and pubertal development, normal LH and T but increased FSH and low E2, micro-orchid testes in inguinal canal, abnormal seminiferous tubules with Sertoli cell-only tubules, atrophy and degenerated epithelium, heterosexual orientation and behavior	Tall stature, persistent linear growth and diffuse bone pain, genu valgum, unfused metacarpal and phalangeal bones and younger bone age (15 yr), osteoporosis, low BMD	Upper normal insulin levels, normal glucose, normal total cholesterol, acanthosis nigricans, BMI 25.4
Bouillon et al. 2004 (82)	Frame-shift mutation causing truncated, inactive enzyme, 17 yr	Congenital hearing problem, high serum T, upper normal range of LH and FSH, undetectable E2 levels, normal testicular volume, sexual and pubertal development	Tall stature, open epiphyses and younger bone age (12 yr), low BMD and bone size	BMI 27.7
Maffei et al. 2007 (434)	Two point mutations, 25 yr	Normal sexual and pubertal development, normal LH and T, FSH slightly elevated, undetectable E2, normal testicular volume, hypospermia, heterosexual orientation and behavior	Tall stature, genu valgum, continuing linear growth, diffuse bone pain, younger bone age (15.3 yr), unfused epiphyses, low BMD, osteoporosis, osteopenia	Obesity, hyperinsulinemia, insulin resistance, dyslipidemia, acanthosis nigricans, nonalcoholic fatty liver disease, hepatomegaly, BMI 35.9

Continued

Table 5.—*Continued*

Original and Related Case Reports	Type of Mutation, Subject Age	Reproductive Effects	Skeletal Effects	Metabolic Effects
Lanfranco et al. 2008 (386)	Compound heterozygous mutation resulting in truncated, inactive aromatase protein, 26 yr	History of right cryptorchidism, normal sexual and pubertal development, slightly elevated FSH, normal LH and T, undetectable E2, normal testicular volume and sperm concentration with slightly reduced motility, normal sexual behavior, heterosexual orientation	Tall stature, genu valgum, unfused epiphyses, osteopenia, low BMD, younger bone age (15.5 yr)	Increased fasting insulin, insulin resistance, dyslipidemia, fatty liver, impaired liver function, acanthosis nigricans, BMI 29.3
Baykan et al. 2013 (47)	Point mutation, 27 yr proband and younger brother	High LH and FSH, normal testosterone, undetectable E2, ambiguous genitalia, normal testicular volume and sperm count, slightly reduced sperm motility	Tall stature, unfused epiphyses, linear bone growth, bone pain, recurrent bone fractures, osteopenia, osteoporosis, younger bone age (15 yr)	High total cholesterol and triglycerides, low HDL, hepatosteatosis, BMI 25.7
Bouchoucha et al. 2014 (81)	Point mutation resulting in reduced aromatase activity, 1–6 yr	Hypospadias and bilateral cryptorchidism, normal hormonal profiles		
Chen et al. 2015 (130)	Compound heterozygous point mutations resulting in decreased aromatase activity, 24 yr	Normal LH, FSH, and T; undetectable E2; normal sexual and pubertal development, sexual behavior, and orientation; normal testes size, sperm count, and viability	Tall stature, genu valgum, unfused epiphyses, osteopenia, younger bone age (16-18 yr), low BMD	Hyperinsulinemia, impaired glucose tolerance, steatohepatitis, dyslipidemia, acanthosis nigricans, BMI 26.5
Miedlich et al. 2016 (464)	Point mutation resulting in truncated inactive protein, 25 yr	Virilization of mother during pregnancy, normal FSH and LH, high-normal T levels, undetectable E2, normal pubertal development, testis size, sexual behavior and libido	Tall stature, bone abnormalities	Normal insulin, glucose, and lipidemia; moderate acanthosis nigricans
		Estrogen receptor 1 mutation		
Smith et al. 1994 (647)	Point mutation resulting in truncated protein, 28 yr	Normal sexual and pubertal development; elevated E2, LH, and FSH; normal T; normal testes size, sperm count, sexual behavior, and orientation; reduced sperm vitality	Tall stature, genu valgum, bone abnormalities, younger bone age (15 yr)	Impaired glucose tolerance and insulin resistance, acanthosis nigricans, BMI 30.5
Bernard et al. 2017 (57)	Point mutation in ligand binding domain with reduced transcriptional activity, 18 yr	Elevated FSH, LH, and E2; low to normal serum T; low serum inhibin B and AMH; unilateral right cryptorchidism; hypoplastic left testis; normal pubertal development	Low bone age (11 yr)	BMI 23.7

with low E2 suffer osteopenia and are fracture prone (206, 208, 241, 726), and decreased bone mass is associated with *CYP19* gene polymorphisms and decreased aromatase activity in men (349).

Hyperinsulinemia and impaired glucose tolerance occur in most men lacking aromatase (130, 386, 433, 434, 647). Furthermore, these patients have increased body mass index and dyslipidemia **(TABLE 5)**, consistent with *Cyp19*KO and *Esr1*KO mice (189, 281, 368, 509). Most AD men have low growth hormone (GH) concentrations, suggesting E2 control of GH, which was recently validated in knockout mouse models where both ESR1 and ESR2 were shown to regulate GH (34).

B. Lack of ESR1 in Men

The reproductive phenotype of men with mutations in their ESR1 that renders it nonfunctional (57, 647) is almost identical to AD men **(TABLE 5)**, with normal testes size and sperm count but reduced sperm viability (191, 293). This mutation is accompanied by high gonadotropins, despite large increases in serum E2 (57). Not surprisingly, E2 did not resolve their clinical symptoms, suggesting an essential role for ESR1 that is not compensated by other ERs (647). Although fertility was not evaluated, it may be impaired due to decreased sperm viability. Furthermore, cross-sectional studies have shown that polymorphisms in exon 4 (LBD) of *ESR1* are associated with idiopathic azoospermia (373, 669) and male infertility (231, 373). Conversely, mutations and polymorphisms in *ESR2* are not associated with infertility (352).

In summary, E2 in men regulates *1*) bone growth, *2*) glucose and lipid metabolism, and *3*) FSH and LH concentrations, while ESR1, but not ESR2, plays an important role in male fertility (reviewed in Refs. 52, 99, 603).

C. Human Cases of Estrogen Excess

Natural cases of excess aromatase activity (EAA) causing estrogen excess in men have been reported. These men have normal male sexual differentiation, pre- or peripubertal gynacomastia, micro-orchidism, accelerated prepubertal growth, advanced bone age, and tall childhood stature. The EAA adults exhibit normal to slightly reduced heights and hypogonadotropic hypogonadism with low to normal LH, FSH, and T and normal to high serum E2 (176, 443). Serum E2/T ratios are elevated (230). However, in contrast to aromatase overexpressing male mice, fertility and spermatogenesis are preserved in EAA men (230, 633).

The EAA condition is transmitted as an autosomal dominant trait (69, 275, 634, 635, 662). Aromatase expression is regulated by its complex tissue-specific promoters and splicing (100). Most men with aromatase overexpression show duplication, inversion, or deletion mutations in *CYP19A1* (230), resulting in overexpressed mRNA and protein activity. Furthermore, aromatase overexpression with high E2 and gynecomastia was reported in boys with rare conditions such as fibrolamellar hepatocellular carcinoma (4), human testicular and ovarian sex cord tumors (100, 144), large-cell calcifying Sertoli cell tumors, and Peutz-Jeghers syndrome (255).

V. ROLE OF ESTROGENS IN NORMAL PROSTATIC DEVELOPMENT AND FUNCTION AND IN PROSTATIC PATHOLOGIES

The prostate gland is derived from the endodermal urogenital sinus, in contrast to the mesodermally derived seminal vesicles, vas deferens, and epididymis. This embryonic origin accounts, in part, for the high rates of aberrant growth and cancer observed in aging prostates, whereas diseases of other male accessory sex glands are exceedingly rare (564). Prostatic development, growth, and function are tightly regulated by androgens, in particular DHT, acting through AR, which also play fundamental roles in prostate cancer progression (564, 764). While not essential, it is recognized that estrogens impact prostate growth, homeostasis, and disease throughout life. These effects are mediated through multiple ERs, including ESR1, ESR2, GPER, and estrogen-related receptors (ERR) that are expressed in a cell-specific manner in prostate **(FIGURE 9)**.

A. Estrogen Actions in Prostate

While low amounts of circulating estrogens are present throughout life in males, during two time periods, in utero development and aging, males are exposed to higher circulating E2, which impacts the prostate gland. During prostate development, estrogens modulate branching morphogenesis and epithelial differentiation through ESR1 and ESR2, respectively (129, 322, 711). However, exposure to elevated endogenous estrogens or variable levels of xenoestrogens (e.g., DES, BPA) can interrupt normal development and predispose to prostatic diseases with aging. Extensive rodent studies involving developmental estrogenization (estrogen imprinting or estrogen reprogramming) have shown that high-dose estrogens during critical developmental windows inhibit prostate growth and drive epithelial and mesenchymal differentiation defects, causing marked structural reorganization (557). Conversely, lower estrogen doses developmentally increase rodent prostate gland bud numbers and adult prostate size, indicating nonmonotonic dose responses (315, 712).

Studies with *Esr1*KO and *Esr2*KO mice determined that these prostatic effects are mediated through stromal ESR1,

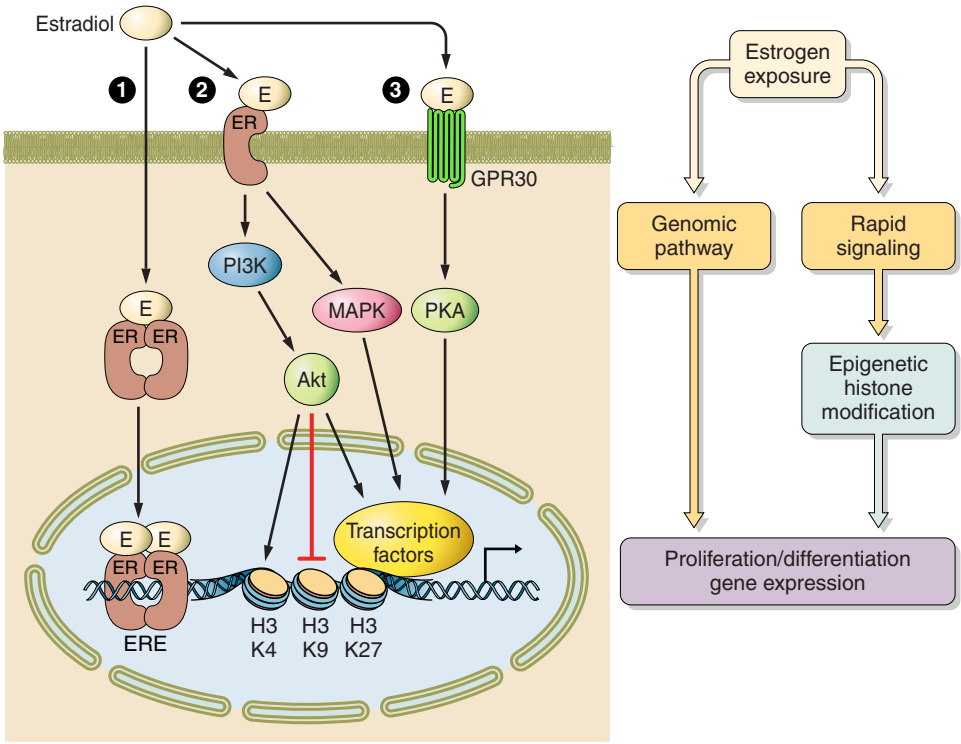


FIGURE 9. Estrogen signaling pathways within prostatic epithelial cells. E2 and other agonists have multiple receptors and pathways that can be engaged to produce a variety of effects within cells. Both ESR1 and 2 (represented as ER) signal through classic genomic pathways. In addition, both ESR1 and 2 are present at the membrane and activate rapid signaling pathways upon ligand binding, including phosphorylation of Akt and/or the MAPK cascade. Multiple downstream effectors can be activated in a context-specific and perhaps ER-selective manner resulting in histone modifications (H3K4, H3K9, H3K27 trimethylation or demethylation) and direct transcriptional activation through intermediaries that include c-*fos*, c-*jun*, SP1, and NFκB as well as phosphorylation of nuclear ERs that amplify their activities. Finally, estrogens can signal through GPER, which activates PKA signaling.

which initiates alterations in prostate steroid receptors and developmental genes (556). Importantly, exposures to natural, synthetic, and environmental estrogens during fetal or neonatal development can lead directly to prostate neoplasia with aging if doses are sufficiently high, and increase susceptibility to hormone-driven carcinogenesis with aging at low doses (560, 565). These life-long changes following brief, early-life estrogenic exposure may result from epigenetic reprogramming of developing prostate cells, leading to altered epigenetic memory at the level of DNA methylation, histone modifications, and noncoding RNAs (301, 674, 720).

Importantly, evidence suggests that similar estrogenic reprogramming occurs in the human prostate **(FIGURE 9)**. Extensive squamous metaplasia in fetal human prostate epithelium is driven by maternal E2 (770). Furthermore, indicators of high estrogen levels during pregnancy, such as high birth weight and jaundice in newborns, are associated with increased prostate cancer risk, whereas indicators of low estrogen levels, such as preeclampsia, are related to decreased risk (194). Directed differentiation of human embryonic stem cells into prostatic organoids in vitro was perturbed by low-dose exposure to the environmental estrogen BPA (103). Furthermore, exposure of adult human prostate progenitor cells to BPA or E2 activated rapid membrane-initiated signal pathways and modified their transcriptome, including SNORDs, a class of noncoding RNAs, through histone methylation reprogramming (301, 559). When human prostate progenitor cells were grafted in mice to form differentiated prostate-like tissue, brief developmental BPA exposure increased susceptibility to estrogen-initiated carcinogenesis in the human epithelium (559). Taken together, these findings support a developmental origin for prostatic disease following early-life estrogen exposures.

Clear evidence shows that elevated adult estrogens are sufficient to drive prostate carcinogenesis, in both animal models and human prostate epithelium (313, 580). This is noteworthy since men are exposed to relatively higher levels of circulating E2 with age. Bioavailable T declines in aging males due to decreased testicular production and increased sex hormone binding globulin levels that combine to lower free circulating T (350). However, circulating free E2 remains constant or rises in aging males due to age-related increases in adipose tissue, which expresses aromatase (708). In addition, estrogens are produced locally by aro-

matase in prostatic stroma (195, 446) and induced in prostate cancer cells, with marked increases in metastatic prostate specimens (466). This results in an increased E2-to-T ratio with aging, allowing a shift towards estrogen dominance, which can occur independent of serum E2 concentrations. Thus increased estrogenic stimulation in aging males may lead to reactivation of prostate growth, neoplastic transformation, and tumor progression.

B. Role of Catechol Estrogens in Prostatic Pathologies

Catechol estrogens are metabolites of E2 and other estrogens, and emerging data suggest that these could also be linked to prostatic pathologies. The most studied of the catechol estrogens are 2-hydroxyestradiol-17β (2-OHE_2) and 4-hydroxyestradiol-17β (4-OHE_2). Catechol estrogen effects do not appear to be entirely mediated through classic ESR1 and ESR2 pathways, although 2-OHE_2 and 4-OHE_2 both have some affinity for ESR1, and stimulate estrogenic responses in some systems and anti-estrogenic responses in reproductive and nonreproductive tissues (reviewed in Ref. 135).

Das et al. (164) showed that uterine effects typically associated with E2, such as increased lactoferrin production and water imbibition, were still seen in *Esr1*KO mice. Furthermore, the ER antagonist ICI 182,780 did not block this process, indicating that these effects were not mediated through ESR1 or ESR2. Subsequent work (547) indicated that there may be specific cytoplasmic binding sites for catechol estrogens that are distinct from ESR1 or ESR2, but these have not been definitively characterized. Recent work in zebrafish indicated that 4-OH-E2 can function as a GPER antagonist and block a critical action of E2 that is normally stimulated through E2 actions through GPER (135), further indicating the complexity of catechol estrogen effects.

Although work with catechol estrogens has predominately utilized female systems, these compounds may be involved in prostatic pathologies. Both 2-OHE_2 and 4-OHE_2 were more potent than E2 in inducing proliferation of a nontransformed prostatic epithelial cell line (BPH-1). In addition, 4-OHE_2 was more potent than E2 in terms of neoplastically transforming these cells. Although data on catechol estrogens are limited, a recent report suggested that urinary concentrations of 2-OHE2 were greater in men than in either pre- or postmenopausal women (134). Thus catechol estrogens may play a role in prostate diseases and have other yet undiscovered actions in males.

C. Expression of ER in the Prostate Gland

ESR1 is primarily localized to prostatic stromal cells in humans, monkeys, dogs, and rodents (90, 555, 618, 620). Studies in rodents have shown relatively high stromal ESR1 expression during perinatal morphogenesis, which significantly declines with puberty as androgens rise, suggesting a specific developmental role (555, 680). Indeed, elegant studies with stromal cell-specific deletion of ESR1 in murine prostates demonstrated that fibroblast ESR1 modulates branching morphogenesis whereas smooth muscle ESR1 regulates stromal cell proliferation and ECM deposition (129, 711). In humans, ESR1 is expressed in stromal cells fetally (2, 626). However, while one report restricts ESR1 protein to only stromal cells (2), another identifies it in periurethral prostatic epithelium during mid-to-late gestation (626). Importantly, squamous metaplasia, observed in all developing human prostates during the third trimester, is directly associated with epithelial and stromal ESR1 in peripheral prostatic acini (626). Recent studies have also identified ESR1 in human prostatic epithelial stem and progenitor cells, where they mediate estrogen-induced stimulation of stem cell self-renewal and progenitor cell proliferation (313, 558, 559), implicating an estrogenic role in maintaining prostate homeostasis through stem cell effects. In disease-free adult prostate, ESR1 is mostly restricted to stromal cells but has been noted in basal epithelial cells (392). Stromal proliferation, a hallmark response to estrogen treatment in most species, is mediated through stromal ESR1, and its increased expression is believed to play a role in benign prostatic hyperplasia (BPH) etiology (367).

In adult rodents, periductal stromal cells express ESR1 (555), enabling estrogen-induced paracrine effects on prostate epithelium. Studies with *Esr1*KO mice demonstrated that developmental estrogen programming of epithelial dysplasia as well as E2-driven squamous metaplasia in adult prostates is mediated through ESR1 (556, 581). While investigations on ESR1 in prostate cancer have produced variable results, possibly a function of disease heterogeneity, ESR1 has been identified in cancerous epithelial cells as well as in cancer-associated fibroblasts (336, 486, 561). Importantly, ample evidence in humans and rodent models identifies a role for estrogens acting through ESR1 to promote prostate cancer growth via stromally mediated factors and recruitment of inflammatory cells, as well as direct actions on tumor cells (see Refs. 366, 486 for review). Of note, ESR1 drives expression of the TMPRSS2-ERG fusion gene in prostate cancer cells, which promotes progression, as well as NEAT1, the most overexpressed lncRNA in human prostate cancer, which in turn epigenetically alters oncogenic target genes (127, 625).

ESR2 was initially cloned from a prostate cDNA library, thus it is not surprising that it plays a role in the prostate, predominantly restraining growth (128, 274, 371). In contrast to ESR1, primarily localized to stromal cells, ESR2 is mainly found in prostatic epithelium **(FIGURE 9)** with limited stromal expression in adult prostate. In developing rat prostate, ESR2 expression is low at birth and increases as

epithelial cells differentiate postnatally (563). In human fetal prostate, ESR2 is widely expressed in epithelial and stromal cells by week 7 and is maintained throughout gestation and postnatally for several months, suggesting a developmental role (2, 626). Furthermore, ESR2 is found in human prostate stem cells, where it restrains their symmetric self-renewal and promotes progenitor cell differentiation (313, 558). Reports vary on ESR2 localization in adult human prostate, with some finding exclusively basal epithelial cell expression (392, 691) while others report high levels in both basal and luminal epithelium (214, 229).

In addition to promoting prostatic epithelial differentiation (437), prostatic ESR2 has been shown to be pro-apoptotic (180, 457), inhibitory to epithelial-mesenchymal transition (EMT) (80), and immunosuppressive (438, 561) based on in vitro and in vivo studies (see Refs. 366, 486 for reviews). Prostatic epithelium also expresses ligand-independent ESR2 isoform variants (β2–5) which can act as either constitutive activators, transcription enhancers, or dominant negative regulators of estrogen action, further complicating estrogenic action within this gland (274, 403, 487). In this regard, the ESR2 (β1 isoform) is gradually hypomethylated and silenced as prostate cancer develops (766), whereas β2 and β5 isoform expression increases and promotes metastasis (402).

Other molecules that mediate estrogen actions are also found within prostate, although their roles are ill-defined. While both ESR1 and ESR2 can mediate membrane-initiated signaling triggered by estrogens in prostate cells in addition to their nuclear transcriptional activity (559), GPER is only at the membrane, where it activates MAPK pathways in response to E2. GPER has been found in normal human prostatic stroma, where it stimulates proliferation through ERK1/2 (529) and in cancer-associated fibroblasts where it regulates ESR1 actions (336). It is also robustly expressed in prostatic epithelial progenitor cells, although its activation or knock-down has limited effects (313). While GPER was found in approximately half of primary prostate cancers, this increased to 80% in metastasized cancers (381). Importantly, in castration-resistant prostate cancers, but not primary tumors, GPER activation triggered cell cycle arrest through the ERK1/2 pathway (409) and inhibited tumor xenograft growth (381), providing a novel pathway for tumor growth regulation. Constitutively active ERR have also been identified in prostate and prostate cancers, where they can modulate proliferative responses induced by ESR1 (130a).

Taken together, evidence shows that there are multiple mechanisms of estrogen action in prostate through several receptors, nuclear and membrane-initiated signaling pathways, and interactions as homo- and heterodimers with both activational and repressive functions. While some pathways trigger growth responses, others restrain growth, drive differentiation, or are pro-apoptotic. Thus estrogen actions in prostate are complex and diverse, which complicates targeting the estrogen axis for control of prostate growth.

VI. NONREPRODUCTIVE FUNCTIONS OF ESTROGENS IN MALES

In addition to male reproductive effects, estrogens also have important actions on many nonreproductive organs, which is a rapidly developing and evolving area of investigation.

A. Adipose Tissue and Metabolism

Many decades of evidence in both humans and laboratory animals show that estrogens are important regulators of white adipose tissue (WAT) in females. Female adipocytes express both ESR1 and ESR2 (155, 537, 714). Ovariectomy of female rodents or other species increases WAT, and estrogen replacement reverses this (reviewed in Ref. 715). Similarly, increased WAT deposition in postmenopausal women is reversed by estrogen replacement (675).

As in females, male WAT expresses ESR1 and ESR2 (155, 536). Other organs associated with satiety, feeding, and body weight regulation, such as hypothalamus and pituitary, also express ESR1/ESR2 in males and females. Males have low but measureable circulating E2 **(TABLE 1)**, but local adipose E2 concentrations could be higher due to aromatase expression in adipose tissue. Despite the established role of estrogen in female WAT, it was unclear for years whether E2 played a role in male WAT.

Over the last two decades, the generation of *Esr1*KO, *Esr2*KO and *Cyp19*KO mice has provided critical information about heretofore unknown and/or unexpected roles of E2/ESR1 signaling in nonreproductive organs. In 2000, work using *Esr1*KO (281, 509) and *Cyp19*KO (338) mice indicated that E2/ESR1 signaling regulated male WAT deposition and various metabolic parameters. Weights of WAT depots in *Esr1*KO males were 100% greater than wild-type controls by 9–12 mo of age. This reflected both adipocyte hyperplasia and hypertrophy (281) and was accompanied by glucose intolerance and insulin resistance (281, 509). Similar effects were seen in *Cyp19*KO males (338), which had obesity and metabolic abnormalities paralleling aromatase-deficient men (434, 469) **(TABLE 5)**. Thus similar increases in WAT and concomitant metabolic changes in both *Esr1*KO and *Cyp19*KO male mice and men lacking aromatase emphasized that E2/ESR1 signaling regulated male metabolism and adipose deposition.

1. Use of conditional knockouts to determine tissue-specific roles of ESR1 on adipose tissue

It was clear for many years that E2 effects on WAT involved direct and indirect actions, but development of conditional

knockouts lacking ESR1 in specific tissues allowed determination of E2 actions in specific tissues. Ovariectomy suppresses overall activity, and thus caloric expenditure, as well as increasing appetite and food consumption, both of which contribute to obesity in *Esr1*KO mice. These effects involve E2 actions in brain regions such as the hypothalamus (reviewed in Ref. 448). Conditional ESR1 deletion in brain led to obesity mimicking that of global *Esr1*KOs (749), indicating a critical role for brain ESR1 in obesity following ESR1 loss.

Male mice with a specific knockdown of ESR1 in adipose tissue showed numerous structural/functional adipose and metabolic changes (166), including increases in adipose markers of inflammation, consistent with global *Esr1*KOs. They also exhibited increased adipocyte size and impaired glucose tolerance. These results indicate that E2 effects on adipose tissue and metabolism are regulated in part by direct effects on adipocytes.

Both ESR1 and ESR2 are expressed in skeletal muscle of humans (737) and other species (346). Both receptors are ubiquitous in muscle, with expression of both ESR1 and ESR2 in myofibers, endothelial cells, and satellite cells (346, 737, 738). Despite ESR1 and ESR2 coexpression in various muscle cell types, work with knockout mice identified unique effects mediated through either ESR1 or ESR2 (45, 97). Endurance training results in increases in both ESR1 and ESR2 in human skeletal muscle, suggesting that their expression is altered by functional demands on muscle (738).

Estrogens are constituents of steroid regimens administered to castrated male cattle to stimulate muscle growth and improve carcass quality (316, 337), indicating that estrogens have anabolic effects on male muscle mass. Similarly, estrogen replacement appears to facilitate skeletal muscle growth in postmenopausal women (644), possibly through effects on satellite cells (347).

Skeletal muscle is responsible for the majority of glucose uptake following insulin stimulation (171, 172). This, in conjunction with ESR1 expression in muscle and anabolic E2 effects on muscle, suggests that effects on muscle could contribute to metabolic impairments of *Esr1*KO mice, consistent with a report that glucose metabolism per kilogram of muscle was 45% higher in women than men (756). A recently developed mouse specifically lacking skeletal muscle ESR1 provided a powerful tool to test this (579). Interestingly, loss of E2/ESR1 signaling only in skeletal muscle of female mice led to obesity and impaired glucose metabolism, insulin resistance, and diminished muscle oxidative metabolism (579), paralleling reports that ESR1 expression is reduced in women with metabolic syndrome (579). A similar relationship was seen in mice, where levels of skeletal muscle ESR1 correlated with insulin concentrations and adiposity.

Is male skeletal muscle function also regulated by E2/ESR1? Although this was not directly addressed, strong correlations between adipose and metabolic effects in male and female *Esr1*KO mice and similar effect in males and females following conditional *Esr1* knockout in other tissues suggest this is likely, although further work is needed.

2. Liver and macrophages

Estrogens regulate lipid, glucose, and cholesterol homeostasis in female mouse liver (222, 631), and transcriptional E2 effects in female liver (526) are primarily through ESR1, the primary hepatic ER in both sexes (370). Male mice with a liver-specific *Esr1* knockout exhibited liver and whole body insulin resistance when consuming a high-fat diet, with altered liver glycogen metabolism and increased fasting plasma triglycerides (765), indicating that the liver is an important target for E2 effects on adioposity and metabolism. Consistent with this, increased E2 concentrations following partial hepatectomy were reported and E2 supported hepatocyte proliferation and regeneration in male mice (695).

Macrophages express estrogen receptors, predominantly ESR1 (473). In recent years, it has become clear that inflammation plays a role in obesity-associated metabolic changes, such as insulin resistance (169). Ribas et al. (578) recently found that female mice with a conditional knockout of ESR1 in macrophages were obese, glucose intolerant, and insulin resistant (578). Males, not yet examined, may show similar effects based on comparable adipose changes in *Esr1*KO (281, 509) and *Cyp19*KO males and females.

3. Adipocyte differentiation

In humans and other species, adult females typically have increased body fat percentages compared with males. This arises pubertally (70), suggesting that despite inhibitory E2 effects on adult WAT, E2/ESR1 can positively regulate developmental adipose differentiation. Recent work from Lapid et al. (389) provides mechanistic insights into this process as well as overall roles of E2/ESR1 signaling in WAT. When ESR1 was knocked out in white adipose progenitors that typically differentiate into subcutaneous fat, these cells preferentially gave rise to alternate brown fat and smooth muscle lineages in males and females. Resultant adults were lean, with improved glucose sensitivity and resistance to weight gain induced by high-fat diets. Thus ESR1 regulates commitment of progenitor cells to WAT in both males and females. These findings, along with earlier findings that estrogen stimulates proliferation of pre-adipocytes (182), may explain the well-known sexual dimorphism in adipose amounts in males and females.

4. Role of ESR2 in adipose tissue

Although ESR2 mRNA is expressed in human and rodent WAT (155), *Esr2*KO mice lack the obesity/insulin resistance of *Esr1*KO and *Cyp19*KO mice (189, 368, 509). Thus ESR1 is most critical for adipose and metabolic effects of E2 in males and females. In addition, mice lacking both ESR1 and ESR2 (189) mimicked adipose/metabolic changes of *Esr1*KO mice, further suggesting ESR1 as the major regulator of E2 effects on WAT.

Despite evidence that ESR2 is not the major regulator of E2 effects on WAT, some work suggests ESR2 can affect glucose tolerance and insulin resistance (44, 45, 221, 478; reviewed in Ref. 448). Similarly, recent work (166) indicated that in the absence of ESR1, ESR2 plays a protective role in suppressing inflammation in adipocytes.

5. Brown and beige adipose tissue in males

Recent work suggests E2 may have effects on brown adipose tissue (BAT), a specialized adipose tissue with a thermogenic role in neonatal heat production. These cells have extensive mitochondria, accounting for their color, and express uncoupling protein 1 (UCP1), which allows uncoupling of oxidative phosphorylation and extensive heat production. A variant of brown fat, beige fat, are cells that are found in WAT. These cells normally express low amounts of UCP-1, but UCP-1 is inducible in these cells (747), which results in concomitant increases in respiration rates. Although rodents have BAT throughout life, humans were thought to only have BAT neonatally. However, recent work described adult human BAT retaining thermogenic properties of neonatal BAT (485). In addition, methodologies to stimulate differentiation of BAT and beige adipocytes in mice have been developed (345, 415), suggesting similar manipulations might be feasible in humans as a weight loss strategy, and this area has become extremely topical because of this. Recent work has indicated that ESR2 may induce preadipocytes to differentiate into BAT (553). Additionally, an ESR2-specific ligand has been shown to increase expression of BAT-specific genes in epididymal WAT, raising the intriguing possibility that ESR2 signaling may increase beige adipocytes in WAT.

Both ESR1 and ESR2 are expressed in male human fetal BAT (706). Expression of ESR1 was more abundant than ESR2, and there was also a unique distribution pattern of these receptors in BAT. Only mature brown adipocytes expressed ESR2, while ESR1 was expressed in mature brown adipocytes, as well as preadipocytes, mesenchymal cells, and endothelial cells within BAT (706). The critical question of whether E2 has effects on male or female brown and/or beige adipocytes will likely attract extensive future interest.

B. Pancreatic Beta Cells

Estrogens have beneficial effects on pancreatic beta cell function and diabetes mellitus incidence. In humans, diabetes mellitus is more common in men (reviewed in Refs. 391, 419). Similarly, in rodent models involving induction of diabetes, females appear to be protected compared with males (391, 419). This is mediated in part by E2 effects on beta cell apoptosis (391), and E2 also has effects on beta cell insulin content, insulin gene expression, and insulin release (17).

Islet cells contain both ESR1 and ESR2 (17, 40, 419, 679), with ESR1 present in greater quantities (17, 40). GPER is also expressed in islets, where it may play a role in E2 effects (40). A critical question here is whether E2 has beneficial effects on male beta cells; existing evidence suggests this is possible. In males, ESR1 is detectable in beta cells (reviewed in Refs. 419, 679). Importantly, chemicals that damage beta cells cause greater damage in *Esr1*KO mice (391), indicating that estrogen plays a protective role in male beta cells. Estrogen may alter beta cell development as well, as shown by estrogen stimulation of proliferation in human islet-derived precursor cells (576). As with E2 effects on beta cells, these effects are ESR1 mediated (576).

C. Bone

Although androgens have significant effects on male bone (653), existing literature indicates that estrogens are more important for bone growth and maintenance (440, 589). Bone cells of men and male rodents express ESR1, ESR2, and GPER (reviewed in Ref. 649). Adult men with mutated ESR1 or aromatase have skeletal problems **(TABLE 5)**, and E2 is essential for normal bone mineralization and mass and bone turnover, but not for linear bone growth, in men (648, 649).

Interventional studies in men using GnRH agonists to suppress endogenous T, aromatase inhibitors, or sex steroid (T and E2) replacement demonstrated that E2 reduced bone remodeling more than T (198). When combined, T and E2 were more effective in maintaining normal baseline bone remodeling than either alone (198). In a recent study, increasing T doses protected from resorptive bone loss and improved bone mineral density (BMD) in androgen-deprived men given a GnRH agonist (208). Importantly, aromatase inhibitors in these men decreased beneficial T effects, suggesting that conversion to E2 is essential for T effects on bone (208). Furthermore, selective estrogen modulators (e.g., raloxifene) reduce bone loss in elderly men (185, 651) or in men undergoing androgen ablation for prostate cancer (650). In addition, aromatase inhibitors are used "off-label" to increase final height in short-stature boys (170, 284, 452). However, long-term health consequences of aromatase inhibitors are not clear. In

male rats, peri-pubertal aromatase inhibitor treatment caused decreased bone strength and altered bone geometry in adults (38).

Osteoporotic men have significantly decreased serum T and E2 (18, 354, 458). Aromatase polymorphisms resulting in decreased aromatase activity in vivo are associated with bone pathology in men (240). A recent epidemiological study positively correlated serum E2 and femoral neck BMD in elderly Chinese men (61). Furthermore, low serum E2 in elderly men increases fracture risk (18, 43, 458). Thus serum E2 concentrations better predict osteoporosis, BMD, and fracture risk than serum T concentrations in elderly men (353, 354, 589, 703).

Male *Cyp19*KO mice have decreased postpubertal femur length growth, lumbar spine BMD, and bone formation (524). Global ESR1 loss decreased bone turnover and cortical bone volume and increased trabecular bone volume in male mice (105, 641, 702). In contrast, loss of ESR2 did not affect bone development or homeostasis (641, 742, 743). Femur length was not affected in either *Esr1*KO or *Esr2*KO mice but was reduced in double-knockout male mice, and this was accompanied by decreased growth plate proliferating zone width (641). This suggests that in the absence of one ER, the other might compensate. Interestingly, *Gper*KO male mice show increased BMD, long bones, and growth plate proliferation, suggesting GPER has negative bone growth effects in male mice (218).

It is noteworthy that rodent bone phenotypes do not completely agree with human phenotypes (649). These discrepancies result in part from species differences, expression of other receptors, and changes in sex steroid hormones. Both *Cyp19*KO and *Esr1*KO mice, but not humans, show increased serum T (440, 649, 740). In addition, bone expresses more AR in *Esr1*KO mice (640). To circumvent changes in sex steroid levels, researchers have studied orchidectomy-induced bone loss in global knockout mice and developed conditional knockout mice with bone cell-specific deletion of ERs and/or AR. Orchidectomy (ORX) of *Esr1*KO and WT mice caused significant trabecular bone loss and decreased cortical bone density (640, 702). Interestingly, T (640, 702) or DHT (471) supplementation completely prevented ORX-induced bone loss in both *Esr1*KO and WT mice (640, 702). Importantly, E2 administration completely or partially prevented ORX-induced bone loss in WT male mice (471, 640, 702). This bone-sparing effect of E2 is primarily ESR1-mediated since E2 was ineffective in *Esr1*KO mice (471, 640, 702).

Osteoclast-specific deletion of ESR1 did not induce bone loss in male mice (481, 694). However, E2 induced osteoclast apoptosis in male mice through ESR1 (481), the main mechanism for bone sparing effects of E2 in females. Osteocyte-specific deletion of ESR1 in male mice decreased trabecular bone volume by reducing both osteoblast and osteocytes and bone formation; however, osteoclast number and cortical bone were unaffected (741). In contrast, ESR1 deletion in an osteoblast-specific lineage did not affect trabecular bone in male mice (694). Furthermore, osteoblast-specific deletion of ESR1 had no effect on bone phonotype in young animals, while at 6 mo trabecular bone volume was decreased (430). Finally, osteoblast-specific deletion of ESR1 decreased cortical bone thickness in young, but not older, adult males (15). Together, these results suggest that cell-specific loss of ESR1 has minimal effect on bone phenotype in male mice.

Many reports indicate that bone-sparing E2 effects involve predominately membrane-initiated signaling through mESR1 both in vitro (5, 363) and in vivo (364, 709). Recent progress in designing estrogens that signal through mESR1 but not nESR1 raises the possibility of treatments that mimic beneficial E2 effects on bone without undesirable side effects on reproductive organs (432). In summary, although there are discrepancies between laboratory animals and humans, E2 is clearly required for normal bone growth and maintenance in men (104) and E2 mediates some T effects on bone homeostasis (353).

D. Cardiovascular System

1. Heart

Cardiovascular diseases (CVDs) are less prevalent in premenopausal women compared with age-matched men, but this protection is lost postmenopausally (575). Cardiovascular protection in women is mediated primarily by E2, and similar effects have been demonstrated in laboratory animals (474). In addition, many differences in cardiovascular functions between men and women develop after puberty, suggesting sex steroid involvement (76, 710).

Males express aromatase (263, 273, 332), ESR1 (263, 420), ESR2 (263, 420), and GPER (178) in their cardiovascular system. In mice, ESR1 is predominantly localized to ventricles, while ESR2 expression is more widely distributed (420). Interestingly, ESR1 is primarily localized in sarcolemma while ESR2 is both nuclear and cytoplasmic (420).

Inhibitory effects of E2 on cardiac hypertrophy are well-known in animal models. Males develop more severe cardiac hypertrophy and fibrosis than females after transverse aortic constriction (646), a cardiac hypertrophy model. This sex-specific difference is mediated through ESR2 (215, 539, 540, 646). Interestingly, *Gper*KO (174) and *Esr2*KO (220) male mice show ventricular hypertrophy by 5 and 6 mo of age, respectively, and E2 attenuates cardiac hypertrophy in male rats (234). Furthermore, *Gper*KO male mice display reduced cardiac function (174). ESR1 and ESR2 are upregulated in left ventricles of men and women with aortic

stenosis (499). Cardiac E2 levels and aromatase expression were decreased in male mice subjected to heart failure, while E2 supplementation restored ejection fractions, prevented cardiac hypertrophy and fibrosis, and promoted angiogenesis (324). Other work has suggested E2 effects on regulation of heart weights and incidence of age-related cardiac fibrosis in male mice (300). Loss of *Esr1* or *Esr2* in mice also did not affect the cardiac-hypertrophic response after pressure overload, although there was reduced hypertrophy in *Esr1*KO mice (646).

Agonists of ESR1 and ESR2 were effective in protecting heart after ischemia/reperfusion (IR) injury in male rats (713); this may be GPER mediated (344). Furthermore, GPER transcripts were severalfold more abundant than ESR1 and ESR2 mRNA in hearts of male mice (344). The pure GPER agonist G1 protects the heart from IR injury and increases recovery rate in males (79, 178, 532, 728). Aromatase overexpression and increased endogenous E2 in males is associated with improved post-ischemia contractile functions (53) but lower basal systolic functions, similar to females (53). In contrast, aromatase deficiency did not affect basal heart functions in males (267). Interestingly, cardioprotective effects of the anesthetic desflurane after IR injury involves induction of aromatase activity in heart, and aromatase inhibition abolished desflurane protective effects and increased myocardial infarct size (332). It appears that cardioprotective effects of E2 after IR injury occur by decreasing reactive oxygen species (380) and reducing intracellular calcium handling (157).

2. Blood vessels

Vasodilation in response to E2 occurs within minutes through nongenomic mechanisms (463). Aromatase is expressed in aorta and endothelial and vascular smooth muscle cells of male mice (332). In men, aromatase inhibition decreased flow-mediated dilation of brachial artery, an indicator of endothelium-dependent vasorelaxation (408). Similarly, flow-induced vasodilation in the brachial artery was absent in a man lacking ESR1 (596, 666, 667) and in *Esr1*KO male mice (668). Furthermore, endothelial-derived nitric oxide production and aortic contraction were decreased in *Esr1*KO males (595). Interestingly, *Esr2*KO males show increased blood pressure (767). E2 effects on vascular dilation include both endothelial-dependent and -independent mechanisms (355) involving prostacyclin and nitric oxide synthesis by regulation of nitric oxide synthase in an ESR2-dependent manner (767). Increased expression of ESR2 was reported in blood vessels after vascular injury in male rats (417).

Hypertension is less common in premenopausal women than age-matched men. However, postmenopausal women develop hypertension at rates equal to or greater than men (276, 574). Anti-atherogenic E2 effects are well known in females and are ESR1 mediated (303, 497). These E2 effects in males can result from direct and indirect actions on lipid metabolism and adipose deposition (497, 689). E2 acts directly on vascular smooth muscle cells to inhibit proliferation and migration in atherosclerosis (159), effects mediated by both ESR1 and ESR2 in males (306). The importance of this effect is illustrated by the finding that a man lacking ESR1 had accelerated coronary arteriosclerosis (596, 666, 667). Furthermore, *CYP19A1* polymorphisms in men are associated with coronary heart disease (51) and hypertension (143). Thus the majority of data in laboratory animal models and estrogen-deficient men suggest that E2 has protective roles in male CVDs.

Some protective cardiovascular T effects are mediated indirectly through E2 (488). Nonetheless, it is unclear from epidemiological studies whether men benefit from increased serum E2 (309, 390, 592). Furthermore, since T is the major source of E2 in men, serum E2 concentrations fluctuate based on T availability and local aromatase and EST activity. Most commonly, lower serum T is more strongly associated with higher risks of death from CVDs in men than serum E2 changes (309, 390, 592). In contrast, higher serum E2 were reported in men with coronary disease (548) and sudden cardiac arrest (483). Thus further research is needed to understand cardiovascular roles of E2 in men.

E. Brain and Behavior

The mammalian brain is a sexually dimorphic organ responsible for gender-specific behaviors. Sex steroids affect behavior by perinatal organizational effects as well as activational effects in adult brains (74, 549, 655). In rodents, fetal Leydig cell T production is essential for male brain differentiation (41). Critically, local T aromatization to E2 is required for masculinization of male brain (41, 451, 479), and thus T effects on brain masculinization are indirect. In the absence of significant T, undifferentiated brains develop as female. In both rats and humans, brain masculinization changes the sizes of several brain structures, including the hypothalamic preoptic area, which is larger in males versus females (326, 670) and controls male-specific sexual behavior in adults (41, 427). Furthermore, sex steroids induce differences in neural circuits between men and women that contribute to gender-specific behaviors (242, 427, 638). Normal male brain differentiation is susceptible to alteration by perinatal exposure to endocrine disruptors (e.g., BPA) (37, 197).

Recently, it has been shown that masculinization of the hypothalamic preoptic area by E2 involves inhibition of DNA methyltransferase (e.g., DNMT3). This decreases the number of methylated CpG sites in masculinizing genes and releases their repression (500). Interestingly, in the same study it was shown that DNA methyltransferase inhibitors were also able to masculinize female brain and induce male sexual behaviors in females (500). This suggests that in the absence of E2 effects on the hypothalamus (as in females),

genes that are responsible for masculinization are suppressed due to increased DNA methyltransferase activity. Furthermore, high sex steroid concentrations occur in neonatal male rodent brains even following gonadectomy and adrenalectomy, suggesting de novo steroidogenesis in neonatal brain (360). Thus brain sexual dimorphism is controlled indirectly by T after local conversion to E2, which then masculinizes the brain (29), although other genes on sex chromosomes may affect male brain development (29, 179, 739). Although conclusive evidence is lacking, a similar process may also drive masculinization of the human male brain.

Aromatase, ESR1, ESR2, and GPER are expressed in male brain (325, 379, 661), and locally produced E2 is considered a brain neurosteroid (46). Masculinizing E2 effects in brain might be mediated through both ESR1 and ESR2 (reviewed in Ref. 369), but a GPER role has not been elucidated.

1. Sexual behaviors

In male rodents, E2 regulates various behaviors (e.g., sexual behavior, aggression, vocalization, learning, and cognition) (41, 152, 427) through rapid membrane-initiated signaling as well as nuclear receptor signaling (151). Neonatal castration and subsequent absence of T (precursor of E2) in rodents reduced adult male sexual behavior (e.g., mounting) and induced female sexual behavior (lordosis) (41, 427). Although *Esr1*KO male mice displayed mounting behavior similar to wild-type controls, intromissions and ejaculations were decreased (507). In addition, *Esr1*KO male mice were less aggressive (507). Administration of T to gonadectomized *Esr1*KO male mice was ineffective in restoring male aggression but restored mounting and intromission (508).

Sexual behavior in *Esr2*KO males is similar to WT controls, although *Esr2*KO mice showed higher aggression and a delayed first ejaculation age (506). Furthermore, E2 administration to castrated *Esr2*KO males induced higher levels of aggression compared with wild-type (498). In contrast, a recently developed exon3-deleted *Esr2*-null male mice showed impaired sexual behaviors (19). However, brain-specific *Esr2* deletion does not affect male sexual behaviors or preoptic area dimorphism in males (484), suggesting that ESR2 is not a major regulator of male sexual behavior. Furthermore, *Cyp19*KO mice showed decreased mounting attempts and intromissions, and no ejaculation in the presence of receptive females (39, 310). This was partially rescued by E2 or DHT (39). Aromatase expression in different parts of the brain controls male aggression (696) and paternal behavior (10). Furthermore, estrogens in female urine stimulate male sexual centers in the brain, relayed through nasal vomeronasal organs (611, 638).

Men lacking aromatase (107, 130, 287, 386, 433, 434, 464, 469) or functional ESR1 (647) reported no change in sexual behavior or orientation, and most had spontaneous erections sufficient for intercourse. However, estrogen influences cannot be completely ruled out since ESR1 are located in brain centers regulating sexual satisfaction and E2 replacement in aromatase-deficient men improves libido and sexual desire in some men (106, 108). Although T supplementation improves libido in hypogonadal men (428), E2 is also beneficial (56, 91, 165), since beneficial T effects on sexual function in men are lost or reduced when T aromatization is inhibited (207, 428). Thus an optimal T/E2 ratio is needed to support normal sexual function in men (613).

2. Nonsexual behaviors

Measurable E2 concentrations are detected in various brain regions (amygdala, hippocampus, cerebral cortex, and cerebellum) in intact adult male rats (119), and gonadectomy reduces brain E2 (42). Administration of E2 to aged male rats improved spatial memory (426). In AR-deficient and WT male rats, E2 increased prefrontal cortex spine growth independent of androgens (e.g., DHT) (269). However, another study showed that E2 decreased dentritic spines in male rat hippocampus (395). In an open-field test measuring anxiety, *Esr1*KO male and WT female mice showed similar behavior (507), while *Esr2*KO and WT male mice showed similar behavior in this test (506). Although T has beneficial mood effects in men, higher E2 concentrations are also associated with less depression (16, 118) and might be mediating T effects by modulating serotonin levels/action in brain (228). Local E2 synthesis occurs in the hippocampus (307), and E2 improves memory in an ESR2-dependent manner in male rats (418). Furthermore, higher serum E2 concentrations are associated with improved spatial memory in elderly men (305) and visual memory in young men (348) while another interventional study showed that E2 is negatively associated with working memory in elderly men (758). In summary, E2 effects on cognitive functions in men are still being established, although some evidence suggests positive effects.

3. Neuroprotective functions after brain injury

In both male and female brain, E2 acts as a neurosteroid (46) and facilitates interneuronal communication (27) through neurite growth and establishment of new neuronal connections (27, 48, 638). Neuroprotective E2 effects in males have been demonstrated following traumatic brain injury (167, 659) and ischemia/reperfusion (stroke) brain injury (120, 721). In addition, it has been reported that aromatase is upregulated in reactive astroglial cells after various types of brain injuries (233). However, in uninjured brains, aromatase is restricted to sexually dimorphic areas of brain (233). Finally, local E2 concentrations increase at brain injury sites (601), again suggesting a neuroprotective role for E2 in male brains.

Protective E2 effects on hippocampal neurons of male mice following excitatory neurotoxicity have been shown using *Cyp19*KO males. Furthermore, aromatase inhibition abolished protective T effects in gonadectomized male mice (35). Treatment with E2 also protects male rat brains after middle cerebral artery occlusion, an experimental stroke model (684). GPER is upregulated in male brain after stroke, and GPER agonists or antagonists worsened or improved, respectively, functional outcomes following stroke (95, 96). Neuroprotective functions of E2 in male brain might be mediated through ESR1, ESR2, GPER1, and other estrogen receptors (reviewed in Ref. 27).

4. Neurodegenerative diseases and learning

Because of its proximity to genes involved in dyslexia (15q21), *CYP19A1* has been considered as a candidate gene for cognitive functions and implicated in reading, speech, and language (20). Furthermore, higher serum E2 concentrations at 5 mo of age are positively associated with language performance in 4-yr-old boys and girls (612). There is a gender-specific preponderance in the onset and predisposition to neurodegenerative and psychiatric diseases such as Alzheimer's and Parkinson's disease, and schizophrenia is more common in males (242, 449), suggesting a possible E2 role in these diseases. In addition, E2 is being considered for therapeutic management of schizophrenia in men (374).

5. Circadian rhythms

Circadian rhythms are regulated by clock genes in brain and peripheral tissues (531). The master circadian clock resides in the brain's suprachiasmatic nucleus, which contains extensive ER in both males and females (531), and E2 has major effects on circadian rhythms and activity in females (11). Recent results suggest estrogens exert similar effects in males (71, 92) through both ESR1 and ESR2 (72, 594). The circadian E2 effects involve both early organizational and adult activational effects (73, 92, 593). Although mechanistic work on circadian clocks has utilized rodents, recent work demonstrated that circadian rhythms and variables regulated by them (e.g., mental function) show marked differences in men and women, suggesting estrogens play critical roles in human circadian rhythms and associated behaviors (604).

F. Effects of Estrogens on the Immune System

Estrogens have major effects on immune system development and function. As for other tissues discussed here, initial studies were on females. However, it has become increasingly obvious that estrogen also regulates male immunity.

1. Estrogen receptor expression in immune organs and cells

Both ESR1 and ESR2 are widely distributed in male and female immune cells (reviewed in Ref. 365), and regulate adaptive and innate immunity. For example, lymphocytes (B cells, $CD4^+$ and $CD8^+$ T cells, NK cells), dendritic cells, and monocytes all express ESR1 and ESR2. In other cell types such as macrophages, hematopoietic stem cells, and myeloid progenitors, ESR1 expression predominates, with ESR2 low or absent (365).

Normal thymic and splenic development depend on ESR1, but not ESR2, in both males and females (416, 755), although thymus expresses ESR1/ESR2 in both sexes (371). Similarly, effects of exogenous administration of estrogen on thymic atrophy in young animals are ESR1 mediated (416).

2. Estrogen function in immune organs and cells

Many major differences in male and female immune function involve estrogen action. Both humoral and cell-mediated immunity are typically more robust in females, but increased immune surveillance in females may predispose to autoimmunity. For example, multiple sclerosis (MS) is three times more common in women than men (7, 8), and disease progression is sexually dimorphic. Despite lower MS incidence, men typically have more rapid MS progression than women following diagnosis (reviewed in Refs. 8, 365). More rapid progression in men may result from neuroprotective E2 effects, which could reduce neurodegenerative processes in females and facilitate tissue repair. The recent study suggesting that disease progression is similar in men and women initially diagnosed at an older age (87) is consistent with the idea of protective estrogen effects only in younger women. Despite apparent protective effects of estrogen in women versus men, it is unclear whether circulating E2 in men modulates rates of overall disease progression or neurodegenerative effects accompanying this disease.

Overall E2 effects on the immune system and its constituent cells are complex and are dependent on estrogen concentration and specific cell types involved, among other factors. In general, E2 regulates a variety of chemokines and cytokines in immune cells such as neutrophils, macrophages, and dendritic cells. For a complete descriptions of E2 effects on immune cells, see Reference 351.

G. Other Estrogen Target Organs and Tissues

1. Urinary system

In male mice, E2 administration following reperfusion injury due to cardiac arrest and cardiopulmonary resuscita-

tion protected kidneys from ischemic injury (319) and E2 protects against age-related kidney changes in male rats (268). Prepubertal castration in male mice decreases kidney weights, while E2 replacement restores kidney size (300). In addition, *Cyp19*KO male mice have increased age-related renal fibrosis (300), and older *Esr2*KO males develop epithelial hyperplasia of the urinary bladder wall, suggesting an ESR2 role in bladder function (368).

Treatment of male mice with a regimen of E2 and T that mimicked the increased ratio of E2 to T in older men produced increased prostatic growth, as well as bladder outlet obstruction and dysfunction of bladder voiding (491). Subsequent work indicated that development of the bladder enlargement that accompanies the E2 + T treatment was mediated through ESR1, indicating that ESR1 may play a significant role in the normal bladder as well as in pathologies that involve altered bladder function.

2. Skin

Skin is an E2 target in males (64), and local estrogen synthesis by androgen aromatization in hair of men has been reported (621). In contrast to E2 effects in females, E2 inhibits wound healing in males through ESR1 (243). However, local E2 administration in elderly men improves wound healing (30). Interestingly, wound healing is faster in castrated young male mice compared with intact males, and systemic E2 or T administration inhibits wound healing (244). Furthermore, E2 regulates epidermal thickness in males by increasing keratinocyte proliferation and inhibits hair follicle growth and cycling in gonadectomized male mice through ESR1 (470).

3. Microbiome

Changes in microbial composition in both feces and seminal vesicles of *Esr1*KO compared with WT mice were recently reported (331). This suggests that microbiomes of male mice, and potentially men, may be regulated by E2.

VII. SUMMARY AND FUTURE DIRECTIONS

The past half-century has witnessed a major paradigm shift in understanding of the role of E2 and other estrogens in the male. Originally considered female hormones, estrogens play critical roles in developing and adult male reproductive organs, especially the efferent ductules where ESR1 is essential for normal fluid reabsorption physiology. In addition, many male nonreproductive tissues express estrogen receptors and are regulated by estrogen. However, many questions remain unanswered, including why the aromatase knockout phenotype does not replicate the *Esr1KO* male. Future studies are needed to understand how AR and ESR1/ESR2, sometimes all expressed in the same cell, work together to regulate cellular activity, as well as how emerging players such as membrane ESR1 and GPER fit into our continually evolving understanding of estrogen's role in males.

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