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Estrogen receptor signaling during vertebrate development

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Abstract

Estrogen receptors are expressed and their cognate ligands produced in all vertebrates, indicative of important and conserved functions. Through evolution estrogen has been involved in controlling reproduction, affecting both the development of reproductive organs and reproductive behavior. This review broadly describes the synthesis of estrogens and the expression patterns of aromatase and the estrogen receptors, in relation to estrogen functions in the developing fetus and child. We focus on the role of estrogens for development of reproductive tissues, as well as non-reproductive effects on the developing brain. We collate data from human, rodent, bird and fish studies and highlight common and species-specific effects of estrogen signaling on fetal development. Morphological malformations originating from perturbed estrogen signaling in estrogen receptor and aromatase knockout mice are discussed, as well as the clinical manifestations of rare estrogen receptor alpha and aromatase gene mutations in humans.

Keywords

Estrogen; aromatase; estrogen receptor; reproductive development; sex differentiation; vertebrate development

Introduction

Estrogens are synthesized in all vertebrates, indicative of a common origin and involvement in important endocrine functions (reviewed in (Eick and Thornton, 2011)). The enzymes of the steroidogenic pathway required to synthesize estrogens from cholesterol can be traced

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back to chordates (Albalat et al., 2011). Throughout evolution, estrogens have been involved in controlling the function of adult reproductive organs and processes. In mammals, estrogen promotes the formation of female secondary sex characteristics, regulates estrous reproductive cycles and affects sexual and maternal behavior. Estrogens also have multiple non-reproductive functions, affecting bone density and strength, blood lipid levels, fat deposition, water and salt balance and brain functions, such as memory. Albeit to a lesser extent, estrogen signaling has important male-specific roles, and it directs certain reproductive functions, such as sperm maturation.

Estrogen also plays significant roles for normal vertebrate embryonic development. The formation of the female reproductive tract has been most studied, however, several reports suggest that estrogens have additional important functions, such as in the development of the male reproductive organs, and sex differentiation of the brain. The main goal of this article is to review the different roles of estrogens during vertebrate fetal development in diverse species, tissues, and developmental stages.

Steroidogenesis

Steroidogenesis is the complex biochemical pathway that involves numerous cytochrome P450 (CYP) and hydroxysteroid dehydrogenase (HSD) enzymes to create steroids from cholesterol. Estrogens formed by steroidogenesis include the three major naturally occurring estrogens: estrone (E1), 17 β -estradiol (E2), and estriol (E3) (Figure 1). The first steps in steroidogenesis convert cholesterol into androstenedione by CYP11A1 and CYP17A1, and 3 β -HSD (HSD3B1) (Figure 1). Following this, E1 is formed by aromatase (CYP19A1). Alternatively, androstenedione is converted to testosterone by 17 β -HSD (HSD17B), and then into E2 by aromatase, although the physiological relevance of this pathway has been questioned (Luu-The, 2013). There are also tissue-specific variations in the synthetic and metabolic pathways of estrogens. For example, during pregnancy E3 is produced in the placenta by conversion from dehydroepiandrosterone (DHEA) and its sulfate (DHEA-S) originating from the fetal and maternal adrenal glands. The activity of the different estrogens is further regulated through sulfate conjugation. A fourth estrogen, estetrol (E4), is produced during pregnancy by the fetal liver. Its synthesis requires two hydroxylases (15 α - and 16 α -hydroxylase), expressed in the fetal liver (reviewed in (Warmerdam et al., 2008)). Other steroidal metabolites with lower estrogenic capacity exist, including 5 α -androstane-3 β , 17 β -diol (3 β -Adiol) and 5-androstene-3 β , 17 β -diol (5-Diol) (Luu-The, 2013). DHEA is one of the most abundant among circulating steroids. It is a metabolic intermediate in the biosynthesis of androgens and estrogens, and it is produced in the adrenal glands and in the gonads. 5-Diol is a metabolite of DHEA (reviewed in (Aidoo-Gyamfi et al., 2009)), and 3 β -Adiol is metabolized from 5 α -dihydrotestosterone (reviewed in (Handa et al., 2008)).

E2 is the most abundant and potent endogenous estrogen in female vertebrates during the reproductive years. The estrogen levels in a woman of reproductive age vary between 30 and 400 pg/mL, with the highest levels near the end of the follicular phase just before ovulation. This estrogen is primarily synthesized in the ovaries by the granulosa cells of the ovarian follicles and corpora lutea. Depending on species, the fluctuations in estrogen synthesis may occur episodically (frogs mating in response to rainfall), bi-weekly (many marine animals),

monthly (humans), semi-annually (cattle), or bi-annually (elephants). In men, small amounts of E2 are synthesized by the Leydig cells in the testes, in the adrenal glands, brain, and fat tissue. The serum levels of E2 in men (10–55 pg/mL) are roughly comparable to those of postmenopausal women (< 35 pg/mL).

As the conversion of androgen to estrogen by aromatase is a rate-limiting step in estrogen biosynthesis, the expression of aromatase is an indication of estrogen production. Many tissues other than the ovary, testis, adrenal gland, and placenta express aromatase in humans, such as the muscle, liver, blood, heart, hair follicles, adipose tissue, bone and brain (Harada et al., 1993; Sayers et al., 2012), suggesting that estrogen has multiple functions in addition to reproduction. In the different tissues, the aromatase transcripts contain varying first exons that are alternatively spliced onto a common site in exon II, and inferring that aromatase expression is driven by tissue-specific promoters that lie upstream of these unique first exons (Kamat et al., 2002). Although the ovaries are the major source of systemic estrogen in pre-menopausal women, the local production of estrogen in peripheral tissues may account for important paracrine-regulated estrogenic functions. The local production of estrogen would be particularly critical for estrogenic actions in men and postmenopausal women, as well as during embryonic development, as discussed below.

Circulating estrogen levels are lower in mice compared to humans, and fewer tissues express aromatase (www.ncbi.nlm.nih.gov/UniGene). Still, aromatase-knockout (ArKO) mice display underdeveloped external genitalia, uteri, and mammary glands, and arrested ovulation in females (Fisher et al., 1998). The males are fertile, but have enlarged male accessory sex glands because of increased content of secreted material.

Estrogen receptors

Estrogens readily diffuse through cell membranes and their cellular functions are mediated by its receptors. Humans and mammals have two ligand-activated transcription factors that bind estrogen, encoded by separate genes, estrogen receptor alpha (ESR1/ER α) and estrogen receptor beta (ESR2/ER β) (Table 1). The estrogen receptors are composed of several domains important for hormone binding, DNA binding, dimer formation, and activation of transcription (Green et al., 1986; Kumar et al., 1986; Warnmark et al., 2003). The DNA-binding domain is highly conserved between the two receptor variants and species (Table 1), suggesting they can bind to similar cis-regulatory chromatin regions. The N-terminal AF-1 domains of the receptors show much less conservation, however, and these differences may contribute to differential recruitment of co-regulators and observed variations in target genes and downstream gene networks (Charn et al., 2010; Grober et al., 2011; Zhao et al., 2010). The ERs' expression patterns and functions vary in a receptor subtype, cell- and tissue-specific manner. Both receptors can form homodimers as well as heterodimerize with each other. E2 activates ER α and ER β with the same affinity although they share only 56% similarity in their ligand binding domains, whereas 3 β -Adiol preferentially activates ER β (Monroe et al., 2005; Papoutsi et al., 2009). The ERs can also be activated through post-translational modifications, and can perform non-genomic signaling (Levin and Pietras, 2008). In addition, the membrane localized G protein-coupled estrogen receptor 1 (GPER1, GPR30) can be activated by estrogens and mediate non-genomic signaling (Liu et al., 2009).

The ERs in birds are highly conserved with the mammalian ones (Table 1). Studies in birds, such as the Japanese quail, *Coturnix japonica*, in which aromatase is expressed in the brain at higher concentrations than in mammals, have shown that male reproductive behaviors are controlled by estrogen (Balthazart et al., 2009). In fish, the receptors are slightly less conserved, especially in the ligand-binding domain (Table 1), and fish can have duplicate copies of the receptors. The genome of the vertebrate zebrafish (*Danio rerio*) codes for three estrogen receptors, ESR1, ESR2a and ESR2b (previously denoted ER α , ER β 2 and ER β 1, respectively) (Menuet et al., 2002). Fish ESR2a and ESR2b likely arose from genome duplication, and have several key amino acid changes compared to human ER β (Hawkins et al., 2000). In the rainbow trout, a second form of ESR1 (ER α -2) has been described and likely exists in other tetraploid teleost species (Nagler et al., 2007). All vertebrates have one copy of the GPER1 receptor (Table 1).

Estrogens present during embryonic development

Estrogens are required both for maternal fertility and for embryo development. For example, treatment of *in vitro* mouse embryo cultures with the anti-estrogen CI 628 or aromatase inhibitor 1,4,6-androstatriene-3,17-dione blocks embryo development (Sengupta et al., 1982; Wu and Doong, 1984). This blockage is alleviated by the co-administration of E2, showing a direct effect of estrogens on early embryo development. The trophoblast cells that eventually develop into the placenta are the first cells during embryo development that have been shown to have aromatase activity, indicative of estrogen production. This activity has been suggested to also constitute a signal of pregnancy to the female body, for example in pig (Bazer and Johnson, 2014). Estrogen is responsible for the proliferation of the uterine endometrium needed for proper implantation of fertilized embryos (reviewed in (Vasquez and Demayo, 2013)). This is primarily mediated by ER α in the uterus, as conditional knockout of ER α in the uterine epithelium results in infertility (Winuthayanon et al., 2010). Aromatase is expressed in the syncytiotrophoblast formed from the fusion of cytotrophoblast cells in humans (Stocco, 2012), which invades the wall of the uterus to establish nutrient circulation between the embryo and the mother. At 4 weeks of equine pregnancy, the embryo proper (ie. the portion of the embryo that becomes the fetus) has capacity to synthesize E1 and E2 and metabolize these estrogens (Raeside et al., 2012). After the formation of the fetal adrenal cortex (at around the ninth week of gestation in humans), the placenta becomes the primary source of fetal estrogen production. Estrogen synthesis in the placenta is achieved through conversion of DHEA and DHEA-S produced by the fetal and maternal adrenal cortex (reviewed in (Kaludjerovic and Ward, 2012)). Near term, 60% of the DHEA and DHEA-S in the female is derived from the fetus. Both ER α and ER β are expressed in the fetal adrenal cortex, and mediate a feed-back system on DHEA production. The fetal adrenal gland is 20 to 30 times larger than its adult counterpart, and after birth it regresses rapidly. Throughout the pregnancy, estrogen mediates an increased size of the uterus and thickening of the uterine wall to accommodate the developing fetus. Although estrogens seem to have specific functions in the developing embryo, the high estrogen levels that rise steadily in the pregnant female due to placental production are shielded off from the embryo by the placenta (Slikker et al., 1982), and through fetal sequestering by alpha-

fetoprotein, protecting the developing embryo from the bulk of maternal circulating estrogens.

During the second trimester, the human fetal liver expresses high levels of several steroidogenic enzymes, including aromatase (O'Shaughnessy et al., 2013). At the same time, the expression of steroidogenic enzymes and steroid receptors in the fetal ovary increases (Fowler et al., 2011) (Figure 2). Immunostaining for aromatase is detected around oocyte nests in pregranulosa cells around primordial follicles and somatic cells, whereas ER β is localized primarily in germ cells. In primates, it has been shown that near-term fetuses deprived of estrogen *in utero* have a reduced number of primordial follicles in the ovaries, which can be restored to normal in animals administered E2 (Billiar et al., 2003). This indicates a role for estrogen during the key period of human primordial follicle formation. Paralleling the requirement of estrogen for the development of female reproductive tissues, estrogen is also needed for normal development of the testis. In primates, estrogen deprivation causes decreased number of spermatogonia (Albrecht et al., 2009). The basement membrane of seminiferous cords are fragmented, the germ cells and Sertoli cells disorganized, and vacuoles present, features which may impair spermatogenesis and fertility in adulthood. In the male fetus, estradiol is produced in the testis. In Leydig cells, the *aromatase* gene is a physiologic target of DAX1 (NR0B1), an orphan nuclear receptor that represses transcription by steroidogenic factor-1 (SF-1), which regulates the expression of many steroidogenic enzymes and other genes engaged in reproduction. *Dax1*-deficient mice have an increased aromatase expression, resulting in Leydig cell hyperplasia, which contributes to infertility in these mice (Wang et al., 2001). As human ejaculated spermatozoa express aromatase, located to the tail and midpiece of spermatozoa, they are also potential sites of estrogen biosynthesis (Aquila et al., 2002).

The importance of estrogen for normal development is highlighted by the ArKO mice. Fetuses of these mice have perturbed development of gonads and mammary glands in females, as described above. Both male and female ArKO mice also display deficits in sexual behavior. In females, this correlates to a decreased population of kisspeptin-immunoreactive neurons in the rostral periventricular area of the third ventricle of the brain (Bakker et al., 2010). Estrogens are locally formed in the mouse brain during development, indicative of an estrogenic function in the developing brain of both females and males. *Aromatase* mRNA is expressed at very early postnatal time points in the mouse brain and in gonads of both sexes (Golovine et al., 2003). In particular, *aromatase* is highly expressed in brain regions involved in reproductive functions, such as the hypothalamus within specific subregions of the neuroendocrine brain (reviewed in (Stocco, 2012)). In addition, *aromatase* is expressed in neurons in the hippocampus, areas of the cerebral cortex, midbrain, spinal cord and cerebellum. Expression has also been detected at pre-synaptic terminals, suggesting a paracrine signaling function of E2 on adjacent cells, or even a neurotransmitter role (reviewed in (Stocco, 2012)).

In *Xenopus*, exposure to estrogenic compounds causes male-to-female sex reversal in gonads. While aromatase is expressed in the gonads, liver, and heart of *Xenopus* (Iwabuchi et al., 2013), the highest expression of aromatase is found in the brain. In fact, aromatase in the brain is at 10-fold higher levels than in the gonads at the sex differentiation stage,

suggesting that brain-synthesized E2 also circulates in the whole body (Iwabuchi et al., 2013). This expression is localized to the choroid plexus, paleocortex, and olfactory bulb.

Also the brains of fish exhibit high levels of aromatase. The zebrafish genome encodes two genes for aromatase, *cyp19a* (*AroA*) and *cyp19b* (*AroB*). *AroA* is predominantly expressed in the ovary, while *AroB* is mainly expressed in the brain in adult zebrafish (Kishida and Callard, 2001). In the forebrain of fish, particularly in the telencephalon, preoptic area, and hypothalamus, the AROB enzyme activity is 100 to 1000 times greater than in the corresponding regions in mammals and birds, although the expression levels vary over the reproductive season (reviewed in (Pellegrini et al., 2005)). AROB is localized to radial glial cells in all brain areas in zebrafish. These cells are involved in neuronal migration but can also through asymmetrical division give birth to neurons, which then migrate into deeper brain layers following radial cell processes. In fish, the radial glial cells persist into adulthood, and may contribute to the capacity of their brains to alter sex-specific pathways throughout the entire life, since many fish species are able to change sex depending on environmental or other influences (reviewed in (Pellegrini et al., 2005)). The mRNAs for both *AroA* and *B* are detected in unfertilized eggs and 1.5 h post fertilization (hpf) embryos, but decline by 12 hpf, which indicates maternal transfer. Between 12–24 hpf, there is a zygotic onset of embryonic *AroA* and *B* transcripts, which continue to accumulate up to 120 hpf, a period corresponding to intense neurogenesis (Kishida and Callard, 2001). The aromatase expression indicates that estrogen is synthesized in the early embryo, and estrogen is known to further upregulate aromatase expression in fish. In addition, the yolk has been shown to contain substantial amounts of maternally-derived estrogen, which could be taken up by the embryo, and be a first source of embryonic estrogen.

ER expression during development

The expression and cellular localization of ER α and ER β can be indicative of the possible effects of estrogens during embryonic development. In the adult human, large-scale sequencing approaches show that *ER α* mRNA is detected in numerous human tissues, with the highest levels in the uterus, liver, ovary, muscle, mammary gland, pituitary gland, adrenal gland, spleen and heart, and at lower levels in the prostate, testis, adipose tissue, thyroid gland, lymph nodes and spleen (Fagerberg et al., 2014; Sayers et al., 2012) (www.ncbi.nlm.nih.gov/UniGene). In the same data sets, human *ER β* mRNA is primarily detected in the lung and testis. *Gper1* is expressed in multiple tissues, with high levels in adrenal gland, nerves, placenta, mammary gland, uterus, lymph nodes and cervix.

The expression of *ER* mRNA in the different developmental stages of oocyte, unfertilized ovum, fertilized ovum, and zygote, morula, blastocyst, egg cylinder, gastrula, organogenesis, and fetus has been analyzed in the mouse using Expressed Sequence Tag sequencing (www.ncbi.nlm.nih.gov/UniGene). Here, *ER α* and *ER β* are found expressed already in the oocyte, the fertilized ovum and zygote, with *ER α* levels also readily detected in the cleavage (the division of cells in the early embryo) and gastrula (Figure 2). *Gper1* expression is not detected until the gastrula stage. This supports that ER α and ER β have functional roles very early during fetal development.

Various human fetal or neonatal tissues have been studied for ER α and ER β expression. Both ERs were found in developing human fetuses using semiquantitative RT-PCR (13 and 20 gestational weeks) and immunohistochemistry (13, 20, and 38 gestational weeks), respectively. Because of variability in the antibody specificity of ER β antibodies in particular, immunohistochemistry results should be interpreted with certain caution. Nevertheless, *ER β* mRNA expression was observed in the adrenal gland, with protein localized to the definitive zone of the adrenal cortex (Takeyama et al., 2001). Low levels of ER β immunoreactivity were also detected in Sertoli cells, spermatogonia and germ cells in the fetal testis and epididymis (Takeyama et al., 2001). In the female reproductive tract, both ER α and ER β were detected in the epithelium of the oviduct (Takeyama et al., 2001). RT-PCR but not immunostaining also detected *ER β* in the fetal brain, heart, lung, and kidney in this study. ER α and ER β immunoreactivities have also been seen in the growth plate of the neonatal human rib bone. Intense staining for ER α was observed in osteoblasts and osteocytes adjacent to the periosteal-forming surface and in osteoclasts on the opposing resorbing surface, in the cortical bone (Bord et al., 2001). In cancellous bone, ER β was strongly expressed in both osteoblasts and osteocytes, whereas only low expression of ER α was seen in these areas (Bord et al., 2001). Nuclear and cytoplasmic staining for ER β was also apparent in osteoclasts. These results demonstrate that estrogenic action in the human fetus is possible at the very early stage after fertilization (zygote), and in the adrenal gland, reproductive tissues, and in the bone (Figure 2).

Studies in mice and rats also show that both ER α and ER β are expressed in distinct patterns in different brain regions during development, in both neurons and glia cells. *ER α* mRNA expression is high in the hippocampus and cortex during the first two weeks of life, whereas *ER β* mRNA expression is present in specific neurons (reviewed in (Wilson and Westberry, 2009)). In the adult brain, ER β is the predominant receptor in neurons of the cortex (reviewed in (Wilson and Westberry, 2009)). Whereas ER α is the main player in sexual differentiation of the brain, ER β has been suggested to affect cortical layering and interneuron migration during early development, and have functions in brain morphogenesis (Fan et al., 2010).

The zebrafish oocyte is maternally loaded with *esr* mRNA, in particular *esr2a* (*ER β 2*) mRNA. This expression disappears between 6 and 12 hpf and returns with the start of the zygotic expression 1 day post fertilization (dpf) (Bardet et al., 2002; Lassiter et al., 2002). Transgenic fish that express green fluorescent protein (GFP) driven by tandem estrogen-response elements, has been constructed to follow estrogen signaling during development and in response to exogenous ER ligands (Gorelick and Halpern, 2011). The expression of GFP in these fish relies on the endogenous expression of the ESRs as well as the availability of ligand. Fluorescence signal is detected in these embryos before 1 dpf (Figure 3). This GFP signal is in accordance with the maternal load described above, as only a cross between a female *Tg(5xERE:GFP)* fish with wild type male DZ fish generates GFP signaling in the early embryo, but not the converse cross between a wt female and a *Tg(5xERE:GFP)* male (Hao et al., 2013). At 1 dpf, the maternal GFP fluorescence fades and the zygotic GFP expression appears mainly in the head region, but only after E2 treatment. In the presence of E2, strong GFP fluorescence is detected from 2 dpf and onwards in the developing liver and

pancreas. Low levels of GFP-expression are also detected in the brain and heart valves at 4 dpf. At 5 and 6 dpf, the GFP expression is clearly visible in the brain, pre-optic area, hair cells, heart valves, liver, pancreas and kidney (Figure 3). In a similar transgenic fish line, estrogen-induced GFP expression is also detected in the muscles of the somites (Lee et al., 2012). The GFP expression in the transgenic fish embryos is induced by exogenous estrogen suggesting that the receptors, but not the ligands, are expressed in liver, pancreas, heart, muscle and brain during early fish development. This can be interpreted to mean that the unliganded (apo-) receptors play roles during development, or alternatively, that the level of estrogen during this time period is too low to result in GFP expression in these transgenic fish.

We have previously mapped estrogen-responsive genes during early zebrafish development by microarray analysis. Through target tissue-enrichment analysis, E2-responsive genes were predicted to originate from the liver, pancreas, kidney and various locations of the brain, which is in accordance with the GFP expression in the ERE-reporter fish (Hao et al., 2013). Genes were also predicted to be regulated in the testis and ovary at 2–4 dpf. Although normal morphogenesis of ovary and testis does not initiate until 10 dpf in zebrafish (Clelland and Peng, 2009; Orban et al., 2009), some genes controlling sex differentiation like *anti-mullerian hormone (amh)* may be altered prematurely by E2-treatment or play other roles in earlier stages of zebrafish development. Similarly to the ERE:GFP transgenic fish, expression of the estrogen-target genes is regulated in the presence of exogenous E2, perhaps indicating an apo-receptor function.

In a recent paper, the ER gene expression in 3–5 dpf zebrafish was mapped using whole-mount *in situ* hybridization (Gorelick et al., 2014). A robust expression of *esr2b* in the liver was demonstrated, and a selective expression of *esr1* is developing valves of the heart identified, thus showing that different ER subtypes are specifically expressed in the heart and liver. A previous report showed that *esr1* is weakly expressed, mainly in the head region at early time points, while *esr2b* is expressed in the liver, pancreas, pectoral fin buds, pronephros, and distinct regions of the brain, and *esr2a* has a ubiquitous expression during gastrulation, which later converts to a head expression (Bertrand et al., 2007). Finally, a third report using *in situ* hybridization of *esr2a* showed that its expression is high in the head, brain region and in proximity to the yolk at 24 to 48 hpf (Celeghin et al., 2011). *esr2a* mRNA was also reported to be expressed during the early-life stage in the epidermis, pectoral n buds, hatching gland and neuromast cells of the lateral line (Celeghin et al., 2011; Froehlicher et al., 2009). This expression of the three different ERs overlaps to a large extent with that of adult fish, where the liver, heart, brain, testis and intestines are major organs of expression (Chandrasekar et al., 2010). Estrogen is known to activate expression of *esr1*, but not *esr2a* and *esr2b*, in the liver (Menuet et al., 2004). GPER1 is expressed in distinct parts of the brain, including the olfactory bulbs and in testis and is abundant in the ovary and muscle (Liu et al., 2009). In adult testis of the fish, *gper1* mRNA is expressed in early germ cells, including the spermatogonia and spermatocytes, as well as in somatic cells such as Sertoli cells (Liu et al., 2009). In the ovary, GPER1 is localized to the oocyte plasma membrane and plays an important role in estrogen regulation of oocyte maturation (Pang and Thomas, 2010).

To summarize, the major expression sites of ERs in mammals during fetal development are the ovaries, testes and adrenal gland. Other tissues with ER expression are the brain, bone, heart, lung, kidney and intestines. In zebrafish embryos, ERs are expressed in liver, pancreas, brain, pectoral fin buds, and pronephros, and at later stages in the ovaries and testes. Oocytes of human, mouse and zebrafish origin are pre-loaded with ER mRNA. Aromatase is expressed in a similar pattern as the ERs in tissues such as the liver, gonads, brain, adrenal gland, heart, lung, kidney and intestines in human fetuses (Price et al., 1992). In mice it is expressed in the gonads and brain, although not all tissues have been investigated. Thus, there are many similarities in the tissue-specific expression of ERs and aromatase between different vertebrate species, indicating that these tissues are highly responsive to estrogenic signaling.

Lessons from knockout mice

Surmising that estrogen activity is important for the development of the tissues and organs in which the estrogen receptors are expressed, knockdown of estrogen receptor expression or estrogen production would be expected to perturb development of these tissues and organs.

Female ArKO mice, that lack production of estrogens, display underdeveloped external genitalia and uteri at 9 weeks of age, as described above. Ovaries contain numerous follicles with abundant granulosa cells and arrested antrum formation before ovulation. No corpora lutea is present. Additionally, the stroma is hyperplastic with structures that appear to be atretic follicles. Development of the mammary glands resembles that of prepubertal females (Fisher et al., 1998). Male mice of the same age show essentially normal internal anatomy, but the male accessory sex glands are enlarged because of increased content of secreted material, as mentioned above. Male ArKO mice are initially capable of breeding and produce litters of average size (Fisher et al., 1998), but progressively develop infertility (Robertson et al., 1999). At that time, spermatogenesis is arrested at early spermiogenic stages, as characterized by an increase in apoptosis and the appearance of multinucleated cells, and there is a significant reduction in round and elongated spermatids. In addition, Leydig cell hyperplasia/hypertrophy is evident, presumably as a consequence of increased circulating luteinizing hormone. The findings indicate that local expression of aromatase is essential for spermatogenesis and provide evidence for a direct action of estrogen on male germ cell development and thus fertility.

The phenotype of the ArKO animals differs in some ways from that of ER knockout mice. In mice, knockout of either *ERα* (ERKO) or *ERβ* (BERKO) does not result in an obvious prenatal phenotype (Lubahn et al., 1993; Krege et al., 1998). However, when *ERα* is deleted both sexes are infertile and mammary gland and testis development is affected (Hess et al., 1997). The male mice have lower sperm count and motility than WT mice and exhibit dilated seminiferous tubules (Hess et al., 1997). ERKO females become infertile because their ovaries harbor large, hemorrhagic, cystic follicles, but no corpora lutea. The females also display uterine hypoplasia. In the BERKO females there is abnormal follicular development and very reduced fertility (Cheng et al., 2002). The double *ERα/ERβ* knockout males are infertile (Couse et al., 1999). They exhibit various stages of spermatogenesis, but the numbers and motility of epididymal sperm are significantly reduced. The double-

knockout females exhibit proper differentiation of the mullerian-derived structures of the uterus, cervix, and upper vagina, but these structures become severely hypoplastic in adults. The ovaries of adult double-knockout females exhibit morphologic phenotypes that are clearly distinct from those of the prepubertal *ERα/ERβ* knockout females and the individual *ER* knockout mice. The double-knockout female ovaries have structures resembling seminiferous tubules of the testis, containing tubule-like structures, degenerating granulosa cells and cells resembling Sertoli cells of the testis. The ovaries of adult *ERα/ERβ* knockout females express *mullerian-inhibiting substance*, *sulfated glycoprotein-2*, and *Sox9*, typical for testis. It has been suggested that the adult *ERα/ERβ* knockout ovary exhibits re-differentiation rather than a developmental phenomenon; origination from a once healthy follicle in prepubertal *ERα/ERβ* knockout ovaries with subsequent age-related increases in the area of transdifferentiation. The loss of both receptors thus leads to an ovarian phenotype that is distinct from that of the individual *ER*-knockout mutants, indicating that both receptors are required for the maintenance of germ and somatic cells in the postnatal ovary.

Estrogen in brain development

There is evidence that the programming of adult male sexual behavior in many vertebrates is largely dependent on estradiol produced during prenatal life and early infancy. Estrogens in the brain play an important role in psychosexual differentiation of rodents, for example, by masculinizing territorial behavior. The same is not true in humans, where the masculinizing effects appear to be mediated exclusively through the androgen receptor. As a result, the utility of rodent models for studying human psychosexual differentiation has been questioned (Rochira and Carani, 2009; Wilson, 2001; Wu et al., 2009); still basic findings of the roles of estrogens in the brain have emerged from studying rodents.

In the rodent brain, immunohistochemistry has shown that *ERα* expression is abundant in the areas associated with reproductive behavior, such as in the hypothalamus, arcuate nucleus, and preoptic area, while *ERβ* is more widely expressed in the paraventricular nucleus of the hypothalamus, in corticotrophin-releasing factor neurons, and may functionally be related to the effects on the hypothalamic-pituitary-adrenal axis (reviewed in (Fan et al., 2010; Walf, 2010)). *ERβ* is also the predominant receptor expressed in the hippocampus, the key brain region regulating complex cognitive and emotional responses. Ovariectomized BERKO female mice are slower than WT females to learn in the Morris water maze. Estrogen addition does not rescue this defect, but rather enhance it. In addition, E2 administration increases the formation of new dendritic spines and excitatory synapses in the hippocampus (reviewed in (Fan et al., 2010)). *ERβ* expression in the brain was recently studied by using bacterial artificial chromosome (BAC) transgenic mice expressing *ERβ* identified by enhanced GFP. This study indicated that *ERβ* expression changes dramatically during postnatal development in several regions throughout the forebrain, and that sex differences in bed nucleus of the stria terminalis principal nucleus and anteroventral periventricular nucleus may be one mechanism through which estrogens can contribute to these sexual dimorphisms in brain and behavior (Zuloaga et al., 2014). Mirroring the expression of ERs, aromatase is also expressed in the fetal brain during development (Harada and Yamada, 1992; Montelli et al., 2012; Stocco, 2012). In the human fetal brain,

aromatase is expressed in the fetal cortex from the end of the fourth gestational month and its expression increases progressively during the third trimester (Montelli et al., 2012).

Overall, estrogen has a neuroprotective function in brain, shown both in *in vitro* cultures, in experimental models of focal and global ischaemia, and in neurodegenerative disease models, such as the Parkinson's disease model (Al Sweidi et al., 2012). In ischaemia models, the protective role of E2 can be counteracted by administration of the ER antagonist ICI 182,780. Both ER α and ER β seem to be involved in the neuroprotective functions. In the developing brain, ER β plays an important role in the regulation of migration of cortical neurons in the upper laminae (reviewed in (Fan et al., 2010)). In BERKO mice, the morphology of the radial glial cells of cerebral cortex is altered compared to wt mice. In the cerebellum, estrogen regulates the dendritic outgrowth in particular of Purkinje cells, evidenced from both *in vivo* and *in vitro* studies. Finally, it has been suggested that ER β affects the morphogenesis of the spinal cord dorsal horn through modulating interneuron development of the superficial lamina (reviewed in (Fan et al., 2010)).

Developmental estrogen-regulated gene expression

Through transcriptomic analysis of estrogen target genes regulated during development, clues can be found as to which biological pathways are regulated by estrogens. Whole mouse or rat embryo analysis of estrogen target genes have not been published to date, but gene expression data of certain tissues are available. A major focus has been to define the role of estrogens in reproductive organ development and sexual dimorphism in the brain.

Microarray analysis of altered gene expression has been performed in the developing uterus and ovaries of Sprague-Dawley rats exposed to the synthetic estrogen 17 α -ethynyl estradiol (EE) from gestational days 11 to 20 (Naciff et al., 2002). The genes regulated by EE in pooled fetal uteri and ovaries are involved in cell growth (e.g. *Ilgf-1*, *Ghr*, *bFgf*, and *neural- and thymus-derived activator for the ErbB kinase*), differentiation (*PrgR*, *retinol-binding protein*, *Nrp-tyrosine kinase*, *dermo 1*), stress response (*Gadd45*, *glutathione S-transferase M5*, *non-neuronal enolase*), and apoptosis (*FSH-regulated protein*, *IL4 receptor*) (Naciff et al., 2002). In another study, the same research group mapped EE-induced genes in the immature uterus/ovaries of the prepubertal rat (Naciff et al., 2005b). Genes that support known EE-induced morphological and physiological changes in the uterus were identified. For example, estrogen increased uterine swelling, which was reflected by upregulation of genes implicated in water and other transport mechanisms in the uterine endometrium, such as *solute carrier family 21 and 26 members*, *secretin receptor*, *kidney androgen-regulated protein*, *mucin 4* etc. Gene products involved in the inflammatory response of the uterus, and corresponding promotion of immune cell infiltration by macrophages and eosinophils were also regulated, including *small inducible cytokine A11 (Scya11)*, *complement component 3 (C3)*, *CD24*, *Fc gamma receptor*, *ficolin B*, *CXC chemokine LIX*, *chemokine-like factor 1*, *cathepsin S*, *lipopolysaccharide binding protein*, *galectin-9*, *complement factor I*, and others. In addition, gene products potentially involved in tissue remodeling, cell growth differentiation and apoptosis were identified and included *mmp7*, *latent transforming growth factor beta binding protein 2*, *syndecan 2*, *tumor necrosis factor induced protein 6* and many more (Naciff et al., 2005b).

Naciff and colleagues also determined gene expression changes induced by EE during fetal development of the rat testis and epididymis. The results showed that several genes were regulated by EE both in testis and in uterus/ovaries. For example, the induction of *Calbindin D*; *IL4R*, *progesterone receptor*, and the repression of *Cyp17* and *Star* were observed in reproductive systems of both females and males. Other genes were only regulated in the testis, including *Dax-1*, *glutathione S-transferase Yc1*, *carboxypeptidase A1*, *phosphatidylethanolamine binding protein*, and *seminal vesicle mRNA for SVS protein* ((Naciff et al., 2005a) and references therein).

In the midbrain, estrogen regulates survival of dopaminergic neurons, and neurotransmitter synthesis and catabolism. Differential display of neuronal cultures from embryonic mouse midbrain has identified estrogen regulation of for example *cyclin D3*, *growth hormone*, *tyroxine hydroxylase*, *Fgf-4*, *presenilin-1*, and the neurotrophin *Bdnf* (Beyer et al., 2003). Early growth-regulating molecules, such as *growth hormone*, were downregulated, while functional proteins for neuronal performance and cell activity, such as kinases and other enzymes, were upregulated, as were genes for neuronal maturation such as *Bdnf* and *Fgf4* (Beyer et al., 2003). Several microarray studies have further been performed, analyzing mouse brains. Mouse hypothalamus estrogen-benzoate regulated genes that function within the extracellular matrix, morphogenesis and development, metabolic and cell proliferation categories (Sakakibara et al., 2013). Further, neurocultures from 8- to 12-week-old human fetal brain tissue containing primary neuronal and glial progenitor cell were subjected to microarray analysis to identify estrogen-regulated genes (Csoregh et al., 2009). The analysis identified genes involved with cell signaling and signal-transduction molecules, chromatin/nuclear/transcription factors, cell development/differentiation, cell growth, proliferation and growth factors, cell death/apoptosis, transporters and ion channels, metabolism, extracellular matrix/cell matrix and cell adhesion, and cytoskeleton.

In the whole organism of zebrafish, we have mapped estrogen-regulated genes in 1–4 dpf embryos (Hao et al., 2013). GO annotation analysis, based on the human homologues of the zebrafish genes, showed a significant enrichment of several biological processes. The E2-regulated functional categories included metabolic process, transcription, transport, and signal transduction. Also, genes for phosphorylation, immune response and multicellular organismal development categories were co-regulated at all time points. Apoptosis and cell proliferation categories were enriched for the differentially expressed genes at 3 and 4 dpf. Clearly, estrogen regulates target genes involved in multiple functions in both the reproductive organs, brains and other tissues.

Sex determination in oviparous vertebrates

While primary sex determination in mammals is strictly chromosomal, exogenous factors can influence the sex differentiation in oviparous birds and fish. In birds, while there is a genetic difference between males and females (genetic females are heterozygotes (ZW) and males are homozygotes (ZZ)), the sex differentiation is strongly regulated by the ratio of androgen/estrogens. Consequently, *in ovo* administration of a sex steroid hormone or an inhibitor of endogenous sex steroid synthesis can cause more or less permanent phenotypical sex reversals ((reviewed in (Bruggeman et al., 2002)). In the absence of exogenous

influences, a Z-localized gene, *Double-sex and Mab-3 Related Transcription Factor 1* (*Dmrt1*), has been implicated in sex determination (reviewed in (Chue and Smith, 2011)). DMRT1 is more highly expressed in male gonads than female gonads, in accordance with males having two Z chromosomes. DMRT1 is not only expressed in birds, but is also highly conserved among mammals, reptiles and fish. In female birds, a lower level of DMRT1 is associated with higher levels of aromatase synthesis, leading to an increased estrogen production at day 5–6 of the 21 days embryonic period. By the 7th day of incubation, *ER*-mRNA is present in the left but not in the right gonad of female embryos. Expression of ERs in the left gonad leads to the development of a cortex and a functional left ovary, while the right gonad regresses. In genetic males, there is also an early ER-mRNA expression, but it is only temporal and disappears by day 10. However, this short expression period of ER in males explains why in birds genetic males can undergo gonadal sex reversal if exposed to exogenous estrogens (reviewed in (Bruggeman et al., 2002)).

In fish, there is a large plasticity of the sex-determining mechanisms. Both genetic and environmental factors, including temperature, or a combination of these factors controls sex determination. As in birds, exogenous exposure to estrogenic compounds may affect the sex ratio of the offspring. Aromatase activity is high during the differentiation of ovaries, and in species with temperature-dependent sex determination, aromatase is expressed in higher quantities at temperatures that yield female offspring (Duffy et al., 2010). Since the genetic component of sex determination in fish and birds is weaker than in mammals, these species are more likely to be adversely affected by environmental exposure to xenoestrogenic compounds, at least when it comes to sex ratios. In contrast to oviparous organisms, the mammalian embryo grows in an estrogenic environment, especially before the formation of the placenta. The estrogen/androgen balance does not determine sex determination in mammals; instead, this task has been taken over by genetic components, such as *sex-determining region Y* (*Sry*). Still, the estrogens affect reproductive organ development and function, and estrogen signaling needs to be strictly controlled during development.

ER mutations in humans

In accordance with its expression pattern, *ERα* polymorphism has been related to bone density and height during late puberty, attainment of peak bone density in young men (Lorentzon et al., 1999), and bone-mass density in women (Becherini et al., 2000). The impact of *ERα* during development is, in humans, further indicated by effects in two individuals, one male and one female, born with homozygous mutations of *ERα* and corresponding estrogen resistance (Quaynor et al., 2013; Smith et al., 1994). While both individuals developed largely normally, effects on bone growth and density, and female reproductive tract development were identified. The male had a homozygous nonsense mutation (R157X) and lacked functional *ERα*. He exhibited incomplete epiphyseal closure, accompanied by continued linear growth into adulthood and a 204 cm tall stature (Smith et al., 1994). His development, including pubertal development and masculinization, appeared normal, while serum E2 and E1 levels were increased, glucose tolerance impaired (Smith et al., 2008), and signs of early atherosclerosis were noted (Sudhir et al., 1997). Also relatives, which carried the heterozygous mutation, showed clear effects on bone growth, mineral content, and structure (Smith et al., 2008). The woman carried a homozygous missense

mutation in the ligand-binding domain (Q375H) of ER α , resulting in an expressed protein that was resistant to estrogen. This resulted in absent breast development, primary amenorrhea, intermittent lower abdominal pain, a small uterus with no clearly identifiable endometrial stripe, markedly enlarged multicystic ovaries, and severe facial acne (Quaynor et al., 2013). At the age of 15.5 years, she had a bone age of only 11 to 12 years and lacked an estrogen-induced growth spurt at the time of puberty. Her serum estrogen concentration was elevated, but glucose tolerance was not impaired. Possibly, the ligand-independent functions of ER α were sufficient to protect against the obesity-metabolic syndrome phenotype, but not to rescue the infertility phenotype (Quaynor et al., 2013), as supported in mouse studies (Clegg and Palmer, 2013). No human mutations for ER β have been described.

Aromatase mutations in humans

Some mutations in the *aromatase* gene can lead to excess aromatase and corresponding increased ER activity. This has been shown to cause early epiphyseal closure and short stature in both sexes (Fukami et al., 2012). Also gynecomastia in boys, and precocious puberty and gigantomastia in girls have been noted. Mutations resulting in aromatase deficiency, accordingly, lead to tall stature, as lack of estrogen does not bring the epiphyseal lines to closure. Females can display aromatase deficiency, sexual infantilism and pubertal failure, primary amenorrhea, ambiguous external genitalia at birth, and polycystic ovaries on pelvic sonography, virilization, and hypergonadotropic hypogonadism (Ito et al., 1993; Morishima et al., 1995). In addition, deficiency of placental aromatase activity can result in virilization of the pregnant mother, as well as pseudohermaphroditism of her female baby (Harada et al., 1992; Shozu et al., 1991 {Morishima, 1995 #64}). The placental aromatase has a critical role in protecting the female fetus from fetal masculinization and the pregnant woman from virilization. Thus, estrogens are further essential not only for sexual development, but also for normal skeletal maturation and proportions, accretion and maintenance of bone mineral density and mass, and control of the rate of bone turnover.

Concluding remarks

We have here described the functions of the ERs during development, focusing on the reproductive tissues and brain. The expression of ERs, in particular ER β , during development is much wider though, indicating additional important tasks for estrogen signaling. Several studies have been published describing the role of estrogen for pancreas and immune system function, among other tissues, in adult animals. It was recently shown that estrogen increases haematopoietic stem cell renewal in pregnant women {Nakada, 2014 #79}. However, the specific functions of estrogen on these tissues during development are less studied, and currently present knowledge gaps that need to be filled.

During development, there is a strict regulation of estrogen. In the case of aberrant estrogen signaling, caused by defective estrogen receptors or dysregulated estrogen production, severe perturbations occur. Aberrant estrogen signaling can also be caused by exogenous exposure to synthetic or xeno-estrogens. For example, the administration of diethylstilbestrol to pregnant women in the 1950's, increased the risk in both female and male babies for multiple reproductive tract developmental abnormalities (Palmlund, 1996).

Thus, to facilitate identification of potential xenoestrogen-induced hazards, an understanding of basic estrogen functions during vertebrate development is required.

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HIGHLIGHTS

- We describe steroidogenesis of E1, E2, E3, E4, 3 β -Adiol and 5-Diol
- The expression of aromatase and ERs is depicted and related to their functions
- Estrogen function through target genes in mice, rats and zebrafish is discussed
- Phenotypes of ER and aromatase knockout mice are compared
- The clinical symptoms of *ER α* mutations in humans are described

Figure 1.

Schematic representation of selected steroidogenic pathways for synthesis of estrogens. Different pathways are present in different tissues. Estrogens are shown in blue, and steroidogenic enzymes in red. DHEAS=Dehydroepiandrosterone sulfate; 5-Diol=5-Androstene-3 β , 17 β -diol; DHEA=Dehydroepiandrosterone; 5 α -Dione=5-alpha-androstane-3,17-dione; ADT=Androsterone, epiADT=epi-Androsterone; DHT=Dihydrotestosterone, 3 β -Adiol=5 α -androstane-3 β , 17 β -diol; E1=Estrone; E1S=Estrone sulfate; E2=17 β -estradiol; E2S=17 β -estradiol sulfate; 3 α Adiol=5 α -androstane-3 α ,17 β -diol; E3=Estriol; E4=Estetrol; Cyp=Cytochrome P450; and HSD=Hydroxysteroid dehydrogenase. Modified from (Labrie et al., 2005) and Warmerdam (Warmerdam et al., 2008).

Figure 2.

Schematic representation of expression of ERs and aromatase during fetal development in mouse, and in the human fetus based on qPCR and immunohistochemistry data. The mouse RNA expression data was extracted from NCBI's Unigene (www.ncbi.nlm.nih.gov/UniGene). The human fetal data is described in publications as referenced in the main text. For the mouse fetus, the Unigene database does not report any expression of aromatase, whereas publications do (Greco and Payne, 1994; Harada and Yamada, 1992). This conflicting information has been marked by +/-.

Figure 3.

GFP expression visualizing estrogen activity in zebrafish embryos from 6 hpf to 6 dpf. First row: Maternal effect of *Tg(5xERE:GFP)* transgenic fish at 6 hpf in the absence of E2. Scale bars, 500 μ m. Second to seventh rows: E2-induced GFP expression in *Tg(5xERE:GFP)* transgenic fish during development. Zebrafish larvae were treated with 1 μ M E2 (in 0.1% DMSO) or vehicle alone (control, 0.1% DMSO) from 3 hpf and imaged at 6 hpf, 1 dpf, 2 dpf, 3 dpf, 4 dpf, 5 dpf and 6 dpf. First column, bright-field images; second column, corresponding GFP fluorescence images; third column, overlay of bright-field and GFP images. 1 dpf, lateral view; 2–4 dpf, dorsal view; 5 dpf, left panel, dorsal, and right panel, ventral view; anterior to the left. Scale bars, 100 μ m. Adapted from (Hao et al., 2013).

Table 1

Conservation of estrogen receptors in vertebrate species as compared to human sequences.

Proteins	(% Identity)					
	M. musculus	G. gallus	C. japonica	X. laevis	O. mykiss	D. rerio
ERα/ESR1	89	79	79	69	54	58
ERα/ESR1 DBD*	100	100	100	99	93	96
ERα/ESR1 LBD*	98	96	95	86	69	67
ERβ/ESR2	86	80	80	70	-	-
ESR2a**	-	-	-	-	57	56
ESR2b**	-	-	-	-	57	56
ERβ/ESR2 DBD	100	100	100	97	-	-
ESR2a DBD**	-	-	-	-	99	99
ESR2b DBD**	-	-	-	-	99	96
ERβ/ESR2 LBD	85	88	88	86	-	-
ESR2a LBD**	-	-	-	-	71	71
ESR2b LBD**	-	-	-	-	72	72
GPER1***	100	87	-	77	-	72

*Abbreviations: DBD (DNA-binding domain), LBD (ligand-binding domain).
 ** Fish genomes encode two paralogs of ESR2. Comparisons are made to the human ERp/ESR2 sequence.
 *** GPER1 gene and protein sequences have only been annotated in the genomes of the species indicated.