Oestrogen receptor knockout mice: roles for oestrogen receptors α and β in reproductive tissues

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Oestrogen is an essential component of female reproduction, with well-characterized functions in the uterus, ovaries, mammary gland and hypothalamic–pituitary axis. The mechanism of oestrogen action involves mediation of the rate of transcription by nuclear-localized oestrogen receptor molecules. Two oestrogen receptors are present in mouse tissues, oestrogen receptors α and β . Each receptor exhibits differential tissue expression patterns. Mouse models with genetically engineered disruption or ‘knockout’ of the oestrogen receptors have been developed. Characterization of the resulting defects in reproductive tissues as well as alterations in physiological and genomic responses has given insight into the receptor-mediated effects of oestrogen in reproduction. Oestrogen receptor α knockout females are infertile because they are anovulatory, have disruption in LH regulation and have uteri that are insensitive to oestrogen. In contrast, oestrogen receptor β knockout females are sub-fertile and primarily lack efficient ovulatory function. Mice with deletion of both oestrogen receptors α and β are similar to those lacking oestrogen receptor α only, but exhibit a unique ovarian pathology. These observed phenotypes elucidate the relative roles of the oestrogen receptors in reproductive functions of female rodents.

Oestrogens are important modulators of normal mammalian reproductive functions. Biochemical, physiological and genetic studies have elucidated a basic mechanism responsible for responses to oestrogens. Circulating oestrogens enter cells containing specific oestrogen receptor (ER) molecules (Fig. 1). In the absence of serum oestrogen, the ER associates with ‘co-repressor’ molecules, which inhibit the transcriptional activity of the ER. Binding to oestrogen causes a conformational change in the ER molecule leading to dissociation of co-repressors and allowing recruitment of ‘co-activators’ (McKenna *et al.*, 1999; McKenna and O’Malley, 2002). Co-activators are molecules that interact with the ER and increase its transcriptional activity, and therefore result in an increased transcription rate of genes that contain ER-binding enhancer DNA sequences (EREs) (Klinge, 2001).

ERs are members of the nuclear receptor (NR) family of transcriptional modulators. NRs modulate transcription by interacting with DNA regulatory sequences that bind discriminately to particular classes of NR as well as with co-activator and co-repressor molecules to regulate the activity of the RNA polymerase complex (Steinmetz *et al.*, 2001; Hermanson *et al.*, 2002; McKenna and O’Malley, 2002). Other examples of NR family members

include additional steroid hormone receptors such as the progesterone and androgen receptors, receptors for vitamins or metabolites such as the vitamin D or retinoic acid receptors, or receptors with no apparent ligand, termed ‘orphan’ receptors.

In the case of the ER, two receptor molecules have been identified. ER α is found in all reproductive tissues, is the most abundant and hence was the first ER to be identified. ER β is the product of a different gene (Kuiper *et al.*, 1996; Tremblay *et al.*, 1997), and bears little similarity to the ER α molecule. However, ER α and ER β both bind oestradiol and interact with ERE sequences. Compounds with preferential binding for either ER α or ER β have also been synthesized, illustrating that the binding activities of ERs can be distinguished (Katzenellenbogen and Katzenellenbogen, 2000; Katzenellenbogen *et al.*, 2001; Meyers *et al.*, 2001; Mortensen *et al.*, 2001; Shiau *et al.*, 2002). Both receptors are also distinct in their transcriptional activities, with ER β showing less potent activity than ER α in most contexts as well as the ability to inhibit ER α activity (Kuiper and Gustafsson, 1997; Paech *et al.*, 1997; Barkhem *et al.*, 1998; Kuiper *et al.*, 1998; Montano *et al.*, 1998; Hall and McDonnell, 1999). ER β expression is most abundant in the mouse in the ovary, uterus, lung, neural tissue as well as male reproductive tissues, although in all of these tissues except the ovary, ER α is more abundantly expressed (Couse *et al.*, 1997).

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Fig. 1. Simplified illustration of the mechanism of oestrogen action in a target cell. Oestrogen can diffuse from the serum into cells. If the nuclear oestrogen receptor (ER) is present, oestrogen will bind and convert the ER to an active conformation. The conformational change causes the dissociation of transcription repressors and the association of transcription co-activator molecules (not shown). Through interaction with specific oestrogen responsive enhancer (ERE) DNA sequences, the active ER modulates the rate of transcription.

Studies using human samples have shown the presence of ER β transcripts in the breast, ovary, testis, uterus, thymus, intestine and kidney (Mosselman *et al.*, 1996; Enmark *et al.*, 1997). In the mouse ovary, ER α is expressed primarily in the thecal and interstitial regions, whereas ER β is primarily expressed in the granulosa cells (Schomberg *et al.*, 1999), indicating that although both ERs are present in the ovary, they are not present in the same cells and therefore are unable to interact directly *in vivo*.

Oestrogen receptor knockout models illustrate roles of ERs in reproduction

Much of our understanding of the physiological roles of oestrogens has come from studies in which circulating oestrogen is depleted by ovariectomy and replaced with exogenous treatments. However, such approaches do not discriminate between responses modulated by ER α , ER β or via another mechanism. The advent of gene deletion techniques has allowed the generation of mice lacking ER α (α ERKO; Lubahn *et al.*, 1993; Couse and Korach, 1999), ER β (β ERKO; Krege *et al.*, 1998) or both ER α and ER β ($\alpha\beta$ ERKO; Couse *et al.*, 1999a), as well as mice that lack the ability to synthesize oestrogens due to deletion of the aromatase gene (ArKO; Fisher *et al.*, 1998). Characterization of the phenotypes exhibited by these models has extended our understanding of the physiological processes governed by oestrogens.

Initial analysis indicates complete infertility in both male and female α ERKO and $\alpha\beta$ ERKO mice, whereas

β ERKO males exhibit normal fertility (Lubahn *et al.*, 1993; Eddy *et al.*, 1996; Krege *et al.*, 1998; Couse and Korach, 1999). β ERKO females overall exhibit decreased fertility, with three subsets of phenotypes in a continuous mating study (Dupont *et al.*, 2000). Some β ERKO females are completely infertile; a second subset exhibits a decreased rate of pregnancies; and a third subset has a normal number of litters but delivers smaller litters.

The decreased or lost fertility prompted further investigation of the requirements for ERs for normal fertility. In the case of females, successful reproduction requires coordinated functions of the hypothalamic–pituitary axis, the ovary, the uterus and the mammary gland. The functions of these components in the various ER knockout models were analysed and compared (Table 1).

Pituitary functions

The pituitary gland, in concert with gonadotrophin releasing hormone pulses from the hypothalamus, is responsible for sensing the endocrine environment and releasing gonadotrophins or lactotrophic hormones. The pituitary hormones must be produced and released in a properly regulated manner for successful oocyte development and ovulation to occur. The production of the gonadotrophins LH and FSH are regulated by a negative feedback mechanism in which the oestradiol surge that occurs at ovulation acts upon the pituitary to negatively regulate GnRH secretion, ultimately leading to a decrease in serum gonadotrophins (Gharib *et al.*, 1990) (Fig. 2). Accordingly, LH mRNA and protein are

Table 1. Components of female reproductive system and phenotypes noted in oestrogen receptor knockout (ERKO) mice

Component	Function	Phenotypes
Pituitary gland	Production and secretion of gonadotrophins	α ERKO and α β ERKO: high LH β ERKO: none
Ovary	Production of progesterone and oestradiol, ovulation	α ERKO: haemorrhagic cystic, high oestrogen and testosterone due to high LH, anovulatory β ERKO: reduced ovulations α β ERKO: lack of ovulation, sex-reversed follicles
Uterus	Proliferation, secretion	α ERKO and α β ERKO: oestrogen insensitive – no growth or induction of oestrogen target genes β ERKO: normal growth and responses to oestrogen
Embryo/uterus	Implantation/decidualization	α ERKO: oestrogen independent decidualization, no implantation β ERKO: normal
Mammary gland	Pubertal development, lactation	α ERKO, α β ERKO: no pubertal development β ERKO: normal development and lactation

Fig. 2. Regulation of mouse ovary by the hypothalamic–pituitary axis. Pulsatile release of gonadotrophin releasing hormone (GnRH) from the hypothalamus regulates release of the gonadotrophins FSH and LH from the pituitary gland. The gonadotrophins act upon the ovary, resulting in recruitment and maturation of follicles. The maturing follicle causes an increase in serum oestradiol, which downregulates GnRH release and the synthesis of LH and FSH. Hypothesized localizations of ER α and β isoforms are indicated.

increased in α ERKO and α β ERKO female pituitary gland (mRNA) and serum (protein), indicating that the infertility in these females may in part have resulted from inability to properly regulate pituitary gonadotrophin concentrations (Couse and Korach, 1999). Concentrations of LH and FSH in the β ERKO female appear to be normal, indicating that ER α is the ER that mediates the negative feedback regulation of LH.

Ovaries

As the proper expression of pituitary gonadotrophins is necessary to stimulate ovulation successfully, it is not surprising that examination of the α ERKO ovaries

reveals lack of fully developed follicles and no apparent corpora lutea. In addition, the ovaries contain many blood-filled cystic structures (Couse and Korach, 1999). This phenotype has also been described in transgenic mice that over-express LH β (Risma *et al.*, 1995), indicating that the appearance of the ovaries in the α ERKO is the result of the exposure to increased concentrations of LH. In agreement with this hypothesis, prevention of LH production in α ERKO mice using GnRH antagonist prevents development of the cystic structures (Couse *et al.*, 1999a). Unlike the α ERKO ovaries, the β ERKO ovaries appear to contain normal follicles at all stages of development as well as corpora lutea (Krege *et al.*, 1998).

The ovaries are the primary source of oestradiol and progesterone. As a measure of this aspect of ovarian function, serum hormone concentrations were assayed in the ERKO mice. Concentrations of progesterone in the α ERKO and β ERKO mice are similar to the average of wild-type cyclic mice, whereas oestradiol concentration is increased in the α ERKO but normal in the β ERKO mouse (Couse and Korach, 1999). α ERKO, β ERKO and control wild-type mice were treated with exogenous gonadotrophins using a standard superovulation protocol to determine whether the ovaries could respond to gonadotrophins. When α ERKO females were treated with exogenous gonadotrophins, only 35% as many ovulations were observed in comparison with control females (Couse *et al.*, 1999b). Similarly, fewer ovulations (18% as many as in the controls) occurred in the β ERKO females treated with exogenous gonadotrophins (Krege *et al.*, 1998). In agreement with the continuous mating study, distinct differences in responses were seen in the superovulation trial in the β ERKO mice. One group did not ovulate, whereas another group could be induced to ovulate but the number of oocytes released was reduced. Examination of the α ERKO and β ERKO ovaries after superovulation showed a reduced number of corpora lutea and a number of un-ruptured antral follicles.

Taken together, these ovarian studies emphasize that the infertility in these ERKO models can be partly accounted for by the inability to induce ovulation. In addition, these observations illustrate that both ER α and ER β are required for ovulation to occur efficiently, although some ovulations were induced in their absence.

$\alpha\beta$ ERKO ovaries

Examination of the ovaries of $\alpha\beta$ ERKO mice indicated a phenotype distinct from those observed in ovaries from α ERKO or β ERKO mice (Couse *et al.*, 1999b). Normal follicles, with a developing oocyte surrounded by granulosa cells were apparent, but follicles with an unusual appearance appeared progressively with age. These follicles lacked an oocyte and contained what appeared to be Sertoli cells, which are normally found in the testis. In addition, intermediate structures with characteristics of both types of follicles, containing a degenerating oocyte and both granulosa and Sertoli-like cells, were also present. Thus, follicles that appear normal, begin to 'trans-differentiate' as the oocytes degenerate, into seminiferous tubule-like follicles. Similar structures have also been reported in the ArKO mouse, which is unable to synthesize oestradiol (Britt *et al.*, 2001). Therefore, lack of both ER α and ER β or the lack of ligand to activate either ER, results in a unique phenotype unlike that of either of the individual ER knockout models. The mechanism leading to the follicular 'trans-differentiation' is under investigation, but is also similar to structures observed in models that lack germ cells (Behringer *et al.*, 1990; Whitworth, 1998). Thus the combined functions of ER α and ER β may be necessary to maintain oocyte integrity and prevent follicular degeneration.

Uterus

The uterus plays a central role in reproduction. In mice, the uterus responds to the ovarian hormones oestradiol and progesterone in preparation for embryo implantation and to the implanting embryo with a massive differentiation of the stromal cell component. Therefore, the development and functioning of the uteri from α ERKO, β ERKO or $\alpha\beta$ ERKO mice were studied as components contributing to infertility. The uteri of both the α ERKO and $\alpha\beta$ ERKO are similarly immature in appearance (Lubahn *et al.*, 1993; Couse *et al.*, 1999b). Thus, although lack of ER α does not prevent normal uterine development, post-pubertal growth is disrupted. In addition, uteri of α ERKO and $\alpha\beta$ ERKO mice do not exhibit sensitivity to oestradiol treatment, as evidenced by: (1) lack of a growth response and (2) lack of induction of target genes, such as lactoferrin and progesterone receptor (Couse *et al.*, 1995). The uteri of β ERKO mice appear comparable to that of a wild type, and respond normally to oestrogen treatment. In addition, although

the β ERKO females exhibit decreased fertility, they are able to carry pregnancies successfully to term, indicating normal uterine function (Krege *et al.*, 1998).

Cross-talk

Oestrogen induces the expression of membrane receptor tyrosine kinase ligands, epidermal growth factor (EGF) and insulin-like growth factor I (IGF-I) in the mouse uterus. In addition, oestrogen treatment results in activation of the IGF-I receptor signalling pathway (Richards *et al.*, 1996). When IGF-I knockout mice are treated with oestradiol, uterine epithelial cells do not undergo mitosis, indicating that activation of the IGF-I pathway is necessary for this uterine response to oestrogen (Adesanya *et al.*, 1999). Conversely, EGF or IGF-I administered in an exogenous dose can induce oestrogen-like responses, such as epithelial mitogenesis, induction of oestrogen target genes and increase in uterine mass (Nelson *et al.*, 1991; Ignar Trowbridge *et al.*, 1992). The mechanism appears to involve ER α , as studies using cells *in vitro* show a requirement for ER α for IGF-I or EGF to induce oestrogen-responsive reporter constructs (Ignar Trowbridge *et al.*, 1992, 1993). These studies have led to a model of 'cross-talk' in which growth factor receptor activation results in maintenance, amplification or induction of ER transcriptional activity even in the absence of circulating oestrogens. α ERKO mice were treated with IGF-I and EGF to determine whether ER α is required for uterine responses to these growth factors. In both cases, the necessary membrane growth factor receptors were present and activated by the treatment (Curtis *et al.*, 1996; Klotz *et al.*, 2002). In addition, *cfos* was induced by EGF in the α ERKO, indicating that the EGFR signalling pathway was fully functional (Curtis *et al.*, 1996). However, no thymidine or bromodeoxyuridine incorporation was induced, indicating that the EGF and IGF-I did not induce a mitogenic response (Curtis *et al.*, 1996; Klotz *et al.*, 2002), and therefore that this response requires ER α . Studies using ovariectomized mice containing an oestrogen-responsive luciferase reporter transgene indicated that IGF-I could induce luciferase activity in the uterus in the absence of oestrogen (Klotz *et al.*, 2002), demonstrating that the IGF-I treatment results in activation of ER transcriptional activity *in vivo*.

Uterine progesterone responsiveness

The α ERKO uterine response to oestrogen, including induction of the progesterone receptor gene (PR) was impaired. Therefore, the ability of the α ERKO uterus to respond to progesterone was also studied. Although the PR gene is not induced by oestrogen in the α ERKO uterus, progesterone receptors are detected by western blot analysis as well as radioligand binding (Curtis *et al.*, 1999). The PR is present in the α ERKO uterus at

about 60% of that present in ER α -containing wild-type controls. Progesterone-responsive genes were induced in the uteri of α ERKO mice, indicating that the PR present was sufficient for normal transcriptional responses (Curtis *et al.*, 1999).

Progesterone is essential in modulating the decidual transformation of the stromal component of the rodent uterus in response to embryo implantation. The decidual transformation of the stromal layer can also be induced with oestrogen and progesterone priming and infusion of oil into the uterine lumen. The ability to elicit a decidual response is commonly used as a measure of progesterone responsiveness. Uterine decidualization was experimentally induced to determine whether the PR in the α ERKO uterus was sufficient to modulate this physiological response. Although this response in mice normally requires oestrogen, decidualization could be induced in the α ERKO uterus (Curtis *et al.*, 1999). Thus, not only is the PR functional for this biological response, the requirement for oestrogen signalling in this response has been relieved.

Although the decidual response is an important component of pregnancy, and was present in the uteri of α ERKO mice, the ability to implant embryos is required to initiate pregnancy. As the α ERKO is anovulatory, the ability of the uterus to implant embryos was studied through experimental transfer of donor embryos into a hormone-primed α ERKO uterus. Although implantations were seen after embryo transfer into hormone-primed control mice, no donor embryo implantations were seen in identically treated α ERKOs, indicating that implantation requires ER α .

Lack of decidual response has been reported in several mouse knockout models. These include mice with disruption in the PR (Lydon *et al.*, 1995), prostaglandin synthetase 2 or Cox2 (an enzyme that synthesizes prostaglandin precursors; Lim *et al.*, 1997), Hoxa10 (a homeobox protein expressed in the developing reproductive tract; Ma *et al.*, 1998), leukaemia inhibitory factor (LIF, an oestrogen-inducible cytokine; Chen *et al.*, 2000) and interleukin 11 receptor (IL-11, a cytokine receptor; Robb *et al.*, 1998). Thus, these genes are all important for a decidual response. In an attempt to determine the mechanism that allows decidualization to occur in the uteri of α ERKO mice without oestrogen priming, the expression of these genes known to be required for decidual response as well as responses induced by intraluminal oil infusion were investigated. These studies indicate the following points. (1) PR, as mentioned above, was expressed in the α ERKO uterus. The hormonal and oil infusion treatments did not result in any differences in PR expression in samples prepared from whole uteri of α ERKO mice as compared with wild-type controls. However, the oil infusion did result in increased PR immunohistological staining in the stroma that was enhanced by oestrogen priming in the wild type, but did not require oestrogen priming in the α ERKO,

indicating that the α ERKO stroma might exhibit increased progesterone sensitivity without oestrogen priming. In addition, the α ERKO tissue exhibited tissue damage, indicating that the oil infusion was more 'traumatic' in the α ERKO than in the wild type (Hewitt *et al.*, 2002). Trauma to the mouse uterus has been reported to allow decidualization to develop in the absence of oestrogen priming (Finn, 1965, 1966). (2) Hoxa10 is expressed identically in wild-type and α ERKO samples (Hewitt *et al.*, 2002). (3) Cox2 expression is rapidly and robustly induced after oil infusion in both wild-type and α ERKO samples. This expression is maintained 24 h after oil infusion, but in the wild type this maintained expression requires oestrogen priming, whereas in the α ERKO the expression is maintained independent of oestrogen (Hewitt *et al.*, 2002). (4) Although LIF is not induced in the α ERKO in response to oestrogen, it is rapidly and robustly induced in wild-type and α ERKO samples after oil infusion. This induction is more robust in the α ERKO samples, which might allow the α ERKO to compensate for the lack of oestrogen induction. (5) Stat3, a transcription factor activated in response to cytokine receptor signalling, is activated rapidly after oil infusion (Hewitt *et al.*, 2002). This indicates that the signalling induced by the oil infusion might involve LIF or other cytokines.

Mammary glands

The phenotype present in the mammary glands of the α ERKO illustrates the role of ER α in mammary gland development. At birth, the mouse mammary gland consists of a rudimentary ductal tree that develops and fills the stroma of the gland in response to increased ovarian steroids at puberty. The α ERKO mammary glands do not grow beyond the rudimentary duct, illustrating the role of oestrogen in ductal elongation (Couse and Korach, 1999). As the α ERKO females do not ovulate, endogenous progesterone concentrations are insufficient to support lobular alveolar development. The rudimentary structure can be induced to develop lobular alveolar structures with progesterone treatment, indicating that the progesterone responsiveness of the mammary ducts is maintained in the α ERKO (Hewitt and Korach, 2000). The β ERKO mammary gland develops normally, and as β ERKO mothers are able to nurse their young, they function normally, indicating a lack of requirement for ER β in mammary gland function (Couse and Korach, 1999).

Conclusion

The phenotypes that result from the deletion of ERs have allowed study of the relative roles of the different ERs in female fertility. In most cases, ER α is the receptor necessary to modulate normal function. However, ER β ablation adversely affects ovarian function. Further

studies will help illustrate the molecular and genomic components involved in the functions of reproductive tissues requiring oestrogen.

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