Ontogeny of the oestrogen receptors ESR1 and ESR2 during gonadal development in the tammar wallaby, *Macropus eugenii*

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Abstract

Oestrogen has wide ranging effects in development mediated mainly via the two oestrogen receptors, α (ESR1, also known as ER α) and β (ESR2, also known as ER β). Oestrogen is the key factor that directs the indifferent gonad to become an ovary in many non-mammalian vertebrates. Oestrogen is not required for early ovarian differentiation in mammals but can disrupt normal testicular development in eutherians. Surprisingly, exogenous oestrogen can cause sex reversal of an XY gonad in two marsupials, the North American opossum and the tammar wallaby. To understand the mechanism by which oestrogen induces sex reversal, we characterised the genes for *ESR1* and *ESR2* and examined their expression during gonadal differentiation in the tammar wallaby, *Macropus eugenii*. Both receptors were expressed in the somatic cells and germ cells of the indifferent gonad in both XX and XY foetuses throughout all stages of development, and persisted in these cells into adulthood. ERs were also present in many other tissues including kidney, pituitary and mammary gland. ER mRNA was not significantly altered by exogenous oestrogen in cultured XY gonads but the receptors translocated to the nucleus in its presence. These findings confirm that there is conserved expression of the ERs in the indifferent gonad despite the lack of available ligand during early gonadal development. The receptors can respond to exogenous estrogen at this early stage and are capable of transducing signals in the early mammalian gonad. However, the selective forces that maintained conserved ER expression in this tissue remain unknown.

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Introduction

Oestrogen receptors (ERs) are members of the nuclear hormone receptor superfamily, contain six functional domains, regions A–F (Kumar *et al.* 1987), and are highly conserved among the vertebrates (Couse & Korach 1999). ERs form homo- and heterodimers and, once activated, these can bind directly to oestrogen response elements (EREs) in target genes to mediate transcription of multiple developmental and homeostatic pathways (Pace *et al.* 1997, Pettersson *et al.* 1997, Lindberg *et al.* 2003). While the primary function of the ERs is as direct transcription factors, they can also indirectly affect gene transcription. When activated by oestrogen, outer cell membrane-bound ERs increase Ca²⁺ and nitric oxide, and signal transduction occurs via MAP kinase pathway (Bjornstrom & Sjöberg 2005, Levin 2005). There are three possible ‘non-classical’ ER pathways: ligand-independent signaling, non-genotrophic effects via a membrane-associated ER and genotrophic ERE-independent signaling via protein–protein interactions with other transcription factors (McDevitt *et al.* 2008).

In mice lacking either ESR1 (also known as ER α) or ESR2 (also known as ER β) or both receptors (double knockout), there is surprisingly little impact on early gonadal development (Couse *et al.* 1995, Korach *et al.* 1996, Dixon *et al.* 1997, Krege *et al.* 1998, Dupont *et al.* 2000). The ESR1 (α ERKO) and double ESR1/ESR2 ($\alpha\beta$ ERKO) knockouts have the greatest morphological and behavioural abnormalities (Lubahn *et al.* 1993, Couse *et al.* 1995, 2001, Eddy *et al.* 1996, Dixon *et al.* 1997, Krege *et al.* 1998, Rosenfeld *et al.* 1998, Couse & Korach 1999, Dupont *et al.* 2000). While early gonadal differentiation appears normal in both female and male mice, reproduction is compromised later in development through a loss of the normal hypothalamic–pituitary–gonadal axis feedback loop (Dupont *et al.* 2000). Targeted disruption of ESR1 results in infertility, while loss of ESR2 causes a later, age-related infertility in females (Couse *et al.* 2000). The phenotype observed in the $\alpha\beta$ ERKO female suggests that loss of oestrogen stimulation and its receptor action result in ovarian transdifferentiation and expression of testicular genes, similar to the phenotype of the aromatase knockout

(ARKO) mouse (Fisher *et al.* 1998, Couse & Korach 1999). Oestrogen administration to the ARKO mouse with transdifferentiated ovaries restores ovarian function and the normal expression of ovarian markers. Both oestrogen and ERs are required to direct and maintain the fate of supporting cells in the gonad, but are not required for their initial specification and differentiation (Couse & Korach 1999, Britt & Findlay 2003, Dupont *et al.* 2003, Britt *et al.* 2004). This is in contrast to the role of oestrogen in birds, reptiles, amphibians and fish in which it is essential in maintaining not only reproductive function but also for sex determination (Mak *et al.* 1983, Pieau *et al.* 1998, Miyashita *et al.* 2000, Pieau & Dorizzi 2004, Katsu *et al.* 2006, Gist *et al.* 2007).

How and why oestrogen became dissociated from sex determination in mammals is unknown. One hypothesis suggests that the development of mammalian (therian) fetuses *in utero* necessitated the change from a hormonal to a genetic sex-determining switch (the *SRY* gene on the Y-chromosome) to afford male fetuses protection against the feminising effects of maternal oestrogen (Mittwoch 1993). However, exogenous oestrogen can cause gonadal sex reversal and ovarian development in a male marsupial young if administered before gonadal differentiation (Burns 1956, 1961, Coveney *et al.* 2001), suggesting that *SRY* offers the foetus no such protection. Furthermore, ERs are expressed in the foetal gonads of mice and humans despite their apparent redundancy at this time, thereby providing a mechanism through which oestrogen could exert its effects if it were present (Greco *et al.* 1992, 1993, Hou & Gorski 1993, Hou *et al.* 1996).

In this study, we report the first comparative study of ERs in a non-eutherian mammal, the marsupial tammar wallaby, *Macropus eugenii*. Marsupials are therian mammals that diverged from eutherian mammals around 148 million years ago (Bininda-Emonds *et al.* 2007). The tammar is a useful model to examine the role of oestrogen and its receptors in foetal development as the majority of sexual differentiation occurs *post partum* (pp) where the young are accessible in the pouch for experimental manipulation (Renfree *et al.* 1992, 1996). The indifferent gonad first forms around day 21 of the 26.5-day gestation, but the first signs of sexual differentiation do not occur in males until the appearance of the testicular cords around day 2 pp. In females, histological differentiation of the ovary is not apparent until around day 8 pp (Renfree *et al.* 1996). Treatment of tammar wallaby young with exogenous oestrogen from the day of birth for 25 days leads to disrupted testicular development (Shaw *et al.* 1988). However, similar treatment of premature young (born on day 25 of gestation) with exogenous oestrogen induces complete XY gonadal sex reversal (Burns 1961, Coveney *et al.* 2001, Renfree *et al.* 2001a). Since the effects of oestrogen are almost entirely mediated through the ERs in the gonad (Couse & Korach 1999), we sought to

characterise the expression of the ERs in the developing tammar wallaby gonad to see if they are present and capable of transducing oestrogen signals during sex reversal. Oestrogen and progesterone receptors have been measured in the female genital tract during pregnancy in adult females (Renfree & Blanden 2000), but nothing is known of ER expression in the gonads during early development or in adult tissues. We therefore examined the ontogeny of expression of ESR1 and ESR2 in the tammar wallaby gonad throughout normal development and during oestrogen exposure.

Results

Molecular characterisation of tammar ESR1 (tESR1) and tammar ESR2 (tESR2) in male and female gonads and adult somatic tissues

Cloning

tESR1 and *tESR2* cDNA was partially cloned by PCR using cross-species primers designed to regions conserved between cow, cat, human, horse, mouse, rat, grey short-tailed opossum, alligator and chicken (Table 1). The full-length cDNA was derived from PCR fragments and the tammar wallaby genome trace archives (www.ensembl.org). *tESR1* and *tESR2* were highly conserved with orthologues from an array of vertebrate species (Table 2).

tESR1 and tESR2 sequence comparison

tESR1 and *tESR2* produced a predicted protein of comparable size to human and other vertebrate species. The greatest size difference was seen in the ligand-binding domain, which is (233aa) 14 amino acids larger in the tammar, and in the F domain, which is 18 amino acids smaller in the tammar. In contrast, *tESR1* had both a

Table 1 Cross species primers designed to regions conserved across cow, cat, human, horse, mouse, rat, grey short-tailed opossum, alligator and chicken.

Primer name	Sequence 5'–3'
<i>ESR1</i>	
1447Fw	ATG ATC AAC TGG GCR(A) AAG AG
1924Rv	CAC ATG ATG AAC AAA GGC AT
383Fw	GTG CCC TAC TAC CTG GAG AA
950Rv	CTC AGC ATC CAA CAA GGC AC
ERS4Fw	AAG CAT TCA AGG GCA TAA CGA
ERS4Rv	ATC TCT TTG GCG TTT ATG TTT
ERS3Fw	CAG GGT GGT AGA GAA AGA TTG
ERS3Rv	TCG TTA TGC CCT TGA ATG GTT
ER1S2Fw	TGC CCT ATT ACT TGG AAA ATG AG
ERS2Rv	CAA TCT TTC TCT ACC ACC CTG
<i>ESR2</i>	
ESRB1FW	TTC CAG TAT GTT CCC TTT AGC AGC
ESRB1RV	GAT TCC ACT CTT TGC GAT AAC
ESRB2RV	AGA AGC ATC AGA AGG TTA GCG
ERB969RV	GAC ATC ATC ATG GAG GCC TC
ERB589FW	GGT CRT GTG AAG GAT GTA AG

Table 2 Sequence homologies of *tESR1* and *tESR2* of the tammar and selected species for which alignments were performed (see Fig. 3). Sequence homologies indicate a high degree of conservation of the *tESR1* sequence with both mammalian and non-mammalian vertebrates. There was a high degree of conservation of the *tESR2* sequence with other mammals and with the chicken.

Species	Accession number	Percentage homology (%)
<i>ESR1</i>		
<i>Bos taurus</i>	NM001001443	68
<i>Equus caballus</i>	NM001081772	85
<i>Felis catus</i>	NM001024231	79
<i>Homo sapiens</i>	NM000125	79
<i>Mus musculus</i>	NM007956	77
<i>Monodelphis domestica</i>	XM001370990	94
<i>Alligator mississippiensis</i>	AB115909	84
<i>Gallus gallus</i>	NM205183	83
<i>ESR2</i>		
<i>Bos taurus</i>	AY785359, BC02418	81
<i>Homo sapiens</i>	AD24432	79
<i>Mus musculus</i>	NM_010157	81
<i>Monodelphis domestica</i>	ENSMOD-T00000011756	94
<i>Rattus norvegicus</i>	NM_012754	80
<i>Gallus gallus</i>	NM_204794	81

smaller ligand-binding domain and F domain than hESR2 (Fig. 1a). Conservation is greatest between ESR1 and ESR2 in the C domain (DNA-binding) which is responsible for the specific recognition of the EREs within the target genes. The A/B domain was the least conserved region between the tERs and compared to other species (Fig. 1b).

tESR1 and *tESR2* mRNA expression in the gonad

tESR1 and *tESR2* mRNAs were expressed in gonads from day 24 of gestation, before the onset of gonadal differentiation in the XY gonad, (around 3 days later) through to adulthood in both males and females (Fig. 2).

Northern blot

ESR1 (1.9 kb) and *ESR2* (1.3 kb) transcripts were detected by northern analysis using a radiolabelled probe. *ESR1* and *ESR2* were detected in ovary, testis, kidney, adrenal, liver, pituitary, hypothalamus and mammary gland tissue (at days 80, 150 and 280 of lactation), with the greatest expression detected in the kidney (Fig. 3).

Western blotting

Western immunoblotting was used to verify the specificity of the (Ab-16 ESR1 and Ab-24 tESR2; Neomarkers, Fremont, CA, USA) antibodies used to determine tESR1 and tESR2 protein staining in the tammar. A single band was observed at ~70 kDa for the tESR1 ovary protein extract, and a single band was observed for tESR2 in the

tammar western blot at ~57 kDa. In the rabbit ovary positive control, two bands were detected for rabbit ESR1 at ~70 kDa and another band between 72 and 80 kDa in the rabbit positive control. No bands were detected in the BSA negative control lane, in the presence of antibody indicating specific binding to the rabbit and wallaby proteins (Fig. 4).

Immunohistochemistry

tESR1 and tESR2 receptor proteins were detected in the tammar wallaby ovary and testis before sexual differentiation, during development and in the adult. There was no sexual dimorphism in the level or distribution of the ERs prior to sexual differentiation. Both receptors were detected in the cytoplasm of supporting cells (i.e. granulosa, theca, Sertoli and Leydig) of male and female gametes until the first signs of male differentiation at day 2 of pouch life (pp) as well as in the cytoplasm and nucleus of germ cells.

In males from day 2 of pouch life, both ERs were observed in the developing seminiferous tubules indicating that protein was mainly confined to the Sertoli and germ cells. Seminiferous tubules were intensely stained in all stages tested indicating that the protein was present throughout development, especially in the cytoplasm of Sertoli cells (Fig. 5j and k). The surrounding stromal tissue was negative for ER staining. Staining of the Leydig cells was largely cytoplasmic but became more intense and nuclear as development progressed (Fig. 6f).

In the adult testis, tESR1 staining was detected at various stages of spermatogenesis. There was no tESR1 immunoreactivity in primary spermatocytes in pachytene, but the cytoplasm of round spermatids was lightly stained and there was little or no nuclear staining (Fig. 6e and f). The strongest staining occurred in the cytoplasm of round spermatids while elongating spermatids showed medium to strong staining (Fig. 6e and f). The peritubular myoid cells surrounding the cords were not immunoreactive for tESR1 (Fig. 6e and f). In the adult testis, the cytoplasm and nucleus of Sertoli and Leydig cells as well as spermatogonia were immunopositive for both ERs (Fig. 6e and f, Table 3).

In females, ER staining was strong in the early developing ovary, mainly in the cortex and localised to the germ cells. Staining was weak or absent from the interstitial tissue of the developing ovary (Fig. 5a, b, d and e). At day 67 (a–c), staining was localised to the germ cells that had aggregated into nests in the cortex and showed both a cytoplasmic and a nuclear distribution. By day 110, the germ cells had organised into primordial follicles surrounded by granulosa cells (Fig. 5d–f), and ERs were localised to the cytoplasm of the developing oocytes (Fig. 5d and e).

In the adult, both receptors were found in the interstitial tissue at all stages of follicle development and in the surface epithelium (Fig. 6a and b).

Figure 1 (a) Diagrammatic representation of the functional domains of human oestrogen receptor alpha (hESR1) compared to that of the tammar. hESR1 is comprised of 599 amino acids (indicated at top) and is organised into six functional domains (A–F). The tammar ESR1 also contains six domains and 595 amino acids. In the human, the A/B region is important for transactivation and gene specificity, C is the DNA-binding domain (DBD), D is the hinge region, which allows the protein to undergo conformational changes, E contains the ligand-binding domain (LBD), and F is thought to play a role in distinguishing between agonist and antagonist binding in hESR1 molecule (for a more detailed review of domain functions see [Couse & Korach 1999](#)). Full-length hESR2 contains 578 amino acids and the tammar 530 amino acids (indicated at top) and has similar functional domain structure (A–F) as hESR1. Region C (DBD) is highly homologous between hESR2 and hESR1 (~96%). Figure adapted from [Pavao & Traish \(2001\)](#). (b) Partial amino acid comparison of tammar ESR1 and ESR2 as determined by ClustalW ([Chenna *et al.* 2003](#)). Conserved amino acids are highlighted in blue and underlined by an asterisk (*), and highly similar amino acids are shown in yellow and underlined by a colon (:), while similar amino acids are indicated by a full stop (.). The highest degree of conservation was found in the C domain (DNA-binding domain; red box, red arrow), which is responsible for the specific recognition of the oestrogen response elements (EREs) within target genes. The ligand-binding domain was also highly conserved (shown by green box, green arrow).

Both ERs were detected in the cytoplasm and nucleus of oocytes at all stages of follicle development (Fig. 6a–d). The granulosa cells of all follicles had predominantly cytoplasmic staining. Both ERs were cytoplasmic in the theca cells (Fig. 6a–d, Table 3).

Effect of exogenous oestrogen exposure

Protein distribution

In XY gonads cultured without oestrogen (control; Fig. 7a–c), the ERs were primarily cytoplasmic and confined to the seminiferous tubules and surface

epithelium, and staining was absent in the interstitial tissue. In the XY gonads cultured with oestrogen (Fig. 7d–f), both ERs became predominantly nuclear and seminiferous cords failed to form as previously described ([Coveney *et al.* 2001](#)).

Quantitative *tESR1* and *tESR2* real-time expression in the XY oestrogen-cultured gonad

Quantitative of XY gonads cultured with oestrogen from day 25 of gestation confirmed expression of *tESR1* and *tESR2* in all samples tested, but there was no significant

Figure 2 RT-PCR of *ESR1* (α) and *ESR2* (β) in the developing and adult tammar wallaby gonads. Expression of both receptors was detected at every stage examined from day 25 of gestation through to adult ovary (Ad Ov) and testis (Ad T). The age of the gonad is shown at the top with d denoting day of gestation and D denoting days *post partum*. Female symbol indicates ovary samples and male symbols indicate testes. C indicates no-template negative controls, and M indicates marker (100 bp ladder; Promega).

downregulation of the receptors after exposure to exogenous oestrogen (d25 of pregnancy cultures: *tESR1* $P < 0.479$ and *tESR2* $P < 0.546$ and D0–2 cultures: *tESR1* $P < 0.099$ and *tESR2* $P < 0.868$; Fig. 8).

Discussion

ERs are widely expressed in marsupial reproductive tissues as they are in eutherian mammals (Kuiper *et al.* 1997). Both marsupial ER genes share a high degree of similarity to the known orthologues from other mammals and vertebrate species. A sexually dimorphic pattern for either *tESR1* or *tESR2* protein or mRNA expression was not apparent in the undifferentiated ovary or testis. However, as the gonads become sexually dimorphic, receptor expression was localised to different regions of the testis and ovary.

Figure 3 Northern analysis of *ESR1* and *ESR2* genes. Each lane contains 10 μ g of total RNA. The northern blot was probed with a labelled *ESR1* and *ESR2* cDNA fragment of 400 and 300 bp respectively. 18S RNA was used to control for loading (bottom panel). The *ESR1* cDNA fragment hybridised to a transcript of ~ 1.9 kb (top panel), while the *ESR2* fragment hybridised to a transcript of 1.3 kb (middle panel). The level of expression varied across tissues for both genes. The kidney had the highest level of expression followed by the brain, ovary, testis and adrenal. Expression for both genes was the lowest in the pituitary and mammary gland.

Figure 4 Western blot of rabbit and tammar ovary protein extracts. A rabbit polyclonal antibody raised against *ESR1* (Ab-16), and a rabbit polyclonal antibody raised against *ESR2* detected a strong signal at ~ 70 and 57 kDa respectively in both the rabbit (positive control) and the tammar ovarian protein samples. Nothing was detected in the BSA (negative control) lane in the presence of antibody, indicative of specific binding to the rabbit and wallaby ER proteins. Sizes of the molecular weight markers (M) are indicated on the left. A large band was detected in the rabbit control covering an approximate range of 72–80 kDa and may represent two bands that have overlapped.

Conservation of the marsupial *ESR1* and *ESR2* orthologues

The cDNA sequences of both *tESR1* and *tESR2* were highly conserved with orthologues from other vertebrate species encode a predicted protein size of 70 and 57 kDa respectively, similar in size to that of other mammals. No splice variants were detected in this study. This is in contrast to other species such as the mouse and human where multiple splice forms exist for both receptors (Fisher *et al.* 1997, Bollig & Miksicek 2000, Stirone *et al.* 2003). *tESR1* was less conserved than *tESR2* when compared to the human orthologues. *tESR2* differed in size across all domains, while *tESR1* differed only in domains E and F suggesting that *ESR2* may be under less selective pressure in mammals.

ERs in the indifferent gonad

As in rodents, both *tESR1* and *tESR2* were detected in the cytoplasm of germ and somatic cells of the gonad of both sexes before sexual differentiation, with some rare nuclear staining. The cytoplasmic localisation of the ERs was expected, as there is no oestrogen present in the gonad at this time (Renfree *et al.* 1992) to activate the receptors and cause their translocation to the nucleus. The occasional nuclear staining observed in some but not all germ cells is unexplained, but could suggest activation of the receptors in ligand-independent or 'non-classical' pathways (Levin 2005, McDevitt *et al.* 2008).

Figure 5 ESR1 and ESR2 in the gonads during development. Both receptors were detectable in the ovary (a–e) and testis (g–k) from early stages of development. In the day 67 pp ovary (a–c), germ cells (G) have clustered together into germ cell nests. ESR1 (a) and ESR2 (b) were detected in the nucleus and cytoplasm of some, but not all, germ cells. Granulosa cells (GC) surrounding the germ cells were also immunopositive for both ERs, with ESR1 staining strongly in both the nucleus, while ESR2 staining was predominantly cytoplasmic. The interstitial tissue was negative. By day 110 pp, the germ cells have become surrounded by granulosa cells to form primordial follicles. Staining was seen for both ESR1 and ESR2 in the cytoplasm of oocytes (O) and was absent from the granulosa cells (GC). Both ESR1 and ESR2 were expressed in the developing indifferent gonad (g–i; day of birth male). ESR1 was more abundant than ESR2, and staining for both receptors was largely cytoplasmic and appeared to be localised to both the somatic and germ cells. In the day 92 pp testis (j–l), ESR1 (j) protein was weakly cytoplasmic in Sertoli cells (SC) but strong and predominantly cytoplasmic in germ cells. ESR2 (k) protein was weakly cytoplasmic in Sertoli cells and strongly nuclear in most germ cells. No staining was evident for either receptor in the peritubular myoid cells (Ptm). Negative controls (c, f, i, l) represent primary antibody preabsorbed with appropriate blocking peptides (NeoMarkers). Scale bars: (a–c, f, l) 100 μ m; (d–e, j–k) 50 μ m; (g–i) 150 μ m.

ERs in the developing and adult testis

As in the mouse, both *tESR1* and *tESR2* genes were expressed in the XX and XY gonads before differentiation, throughout development and in adulthood (Greco *et al.* 1992, 1993). However, even if ERs are activated by other signals, they are clearly not essential for development since the receptors can be deleted without disrupting sexual development in the mouse (Couse *et al.* 1995, Korach *et al.* 1996, Mosselmann *et al.* 1996, Dixon *et al.* 1997, Krege *et al.* 1998, Dupont *et al.* 2000). In the tammar, the testis cords form by day 2 pp (Renfree & Short 1988, Renfree *et al.* 1995, 1996, 2001b, 2006), and both ERs are localised to the Sertoli and germ cells. These observations are similar to those reported for rat ESR2 in which it occurs in multiple cell types, but different from the rat ESR1, which is confined to the Leydig cells (Saunders *et al.* 1998).

By day 4 pp, both receptor proteins were also present in the Leydig cells as in the cat and dog but different to mice and rats (Jefferson *et al.* 2000, Nie *et al.* 2002). Peritubular myoid cells in the tammar testis were negative (Fig. 6e and f). This pattern remained the same throughout the lengthy period of development in the pouch and in adults. The location and intensity of ER staining varied with age in a similar manner to those of the rat, mouse, human and other primates (O'Donnell *et al.* 2001).

Sertoli and Leydig cells are important sources of oestrogen in adult mammals, and these two cell types play an important role in aromatisation (Eddy *et al.* 1996). Tammar Sertoli and Leydig cells synthesise and express both ER receptors, similar to the mouse, suggesting a conserved role for oestrogen in the male reproductive tract (Tsai-Morris *et al.* 1986, Eddy *et al.* 1996, O'Donnell *et al.* 2001). Oestrogen also affects

Figure 6 ESR1 and ESR2 in adult ovary (a–d, g) and testis (e, f, h). In the adult female, both ESR1 (a) and ESR2 (b) were nuclear and cytoplasmic in the oocyte (O) at all stages of folliculogenesis, and were particularly strong in the primordial follicles (PrF). Granulosa cells (GC) were predominantly immunopositive in the cytoplasm of all follicles (except primordial follicles) but became nuclear in some cells of primary (1°F), secondary (2°F), and antral follicles (AF). The surface epithelium (SE) was stained for ESR2. (c) ESR1 was weakly cytoplasmic in the theca cells (Th) of antral follicles. (d) ESR2 was predominantly cytoplasmic in granulosa and weakly staining in the theca cells of secondary follicles. (e) ESR1 was detected prominently in the nucleus of the spermatocytes (Spc). It was weak and cytoplasmic in the cytoplasm at various stages of sperm development: spermatogonium (Spg). Weak staining occurred in Sertoli cells (SC). There was no obvious staining in the sperm tails (SpT) or heads (SpH). The peritubular myoid cells (Ptm) surrounding the cords were not immunoreactive for ESR1 or ESR2. (f) ESR2 in the adult testis was cytoplasmic in Sertoli cells and primary spermatogonia (Spg), and staining was predominantly cytoplasmic in the spermatocytes (Spc) and was cytoplasmic in the sperm head (SpH). Leydig cells (LC) were strongly stained in both the cytoplasm and nucleus. Negative controls with primary antibody conjugated with blocking peptides are shown for ESR1 (g) and ESR2 (h). Scale bars: (a–d) 200 µm; (e) 100 µm; (f) 50 µm; (g) 200 µm; (h) 100 µm.

Sertoli cell division and has an inhibitory effect on Leydig cell proliferation early in development in the rat (Abney & Myers 1991, Dorrington *et al.* 1993, Sharpe *et al.* 1998, Atanassova *et al.* 1999). tESR1 and tESR2 localisation persisted in Sertoli and Leydig cells in the adult testis, which is consistent with an ongoing role for oestrogen in testicular function (Eddy *et al.* 1996, Hess *et al.* 1997, 2001). The co-expression of tESR1 and tESR2 in the developing tammar testis also suggests a conserved role for mediation of oestrogen action through the formation of heterodimeric complexes or a role for ESR2–ESR1 co-regulation in these cells (Lindberg *et al.* 2003).

Gonadotrophins from the pituitary initiate and maintain spermatogenesis (Ciocca & Roig 1995). This is mediated by oestrogen feedback in the hypothalamic–pituitary axis. *ESR1* and *ESR2* mRNAs were detected in the tammar hypothalamus and pituitary. Therefore, it is likely that a similar mechanism of oestrogen negative feedback occurs in the hypothalamic–pituitary axis in tammar. tESR1 and tESR2 were also found in germ cells at all stages of development, which is consistent with a role in oestrogen-mediated germ cell maintenance and anti-apoptotic ESR1 receptor-mediated cell survival (Couse & Korach 1999, Pentikainen *et al.* 2000). The expression of tESR1 and tESR2 in spermatozoa is similar to that in humans (Durke *et al.* 1998, Aquila *et al.* 2004) in whom oestrogen stimulates sperm motility (Lubahn *et al.* 1993, Korach 1994, Eddy *et al.* 1996, Hess *et al.* 1997) and lactate production via ESR1 (O'Donnell *et al.* 2001). In humans, ESR2 is involved in mediating oestrogen action in mitochondria, calcium influx modulation, ATP production and free-radical species generation (Solakidi *et al.* 2005).

ERs in the developing and adult ovary

As development proceeds in the tammar ovary, germ cells aggregate into clusters and divide mitotically within the cortex up to day 25 pp, when the germ cells begin to enter meiosis (Alcorn & Robinson 1983). There was a notable increase in both receptor proteins during this phase, but this was more pronounced for tESR2 than tESR1. Similar observations have been noted in the hamster where both receptors are expressed long before the ovary begins to develop morphologically distinct structures (Yang *et al.* 2004). From late development (from day 110 pp) through to adult, both receptors were found strongly expressed in the primary oocytes. This is around the time of the onset of folliculogenesis and initiation of oestrogen production in the tammar (at least by 150–250 days pp; Tyndale-Biscoe & Renfree 1987). Since the initial formation of primary oocytes occurs in an environment in oestrogen, the subsequent appearance of oestrogen may negatively regulate LH and

Table 3 Distribution of ESR1 and ESR2 in the adult testis and ovary.

Testis	ESR1	ESR2
Peritubular myoid cells	—	++
Sertoli cells	+++/c	+++/c & n
Leydig cells	+++/c & n	+++/c & n
Spermatocytes	+++/c & n	+++/c & n
Spermatids		
Round	+++/c	+++/c
Elongated	+++/c	+++/c
Spermatozoon (tail)	—	+++
Ovary	ESR1	ESR2
Interstitial tissue	+++/c & n	+++/c & n
Oocytes		
Primordial	+/c	+/c
Primary	+/c	+/c
Antral	+/c	+/c
Granulosa cells		
Primordial	+/c	+/c
Primary	+/c	+/c
Antral	+/c	+/c
Theca cells		
Primary	+/c	+/c
Antral	+/c	+/c
Stroma	—	—
Germinal epithelium	+/c & n	+++/c & n

Staining intensity was scored on a scale of 1–3 with 1 representing light staining and 3 representing dark staining. (c) denotes cytoplasmic staining and (n) denotes nuclear staining. The absence of staining is denoted by (—).

FSH via tESR1 in the hypothalamic–pituitary axis and enhance folliculogenesis via tESR2 in the ovary, as it does in other mammals (Emmen *et al.* 2005, Glidewell-Kenney *et al.* 2007). Both receptors are present in tammar Graafian follicles that contain rising levels of oestrogen (Harder *et al.* 1985) and may be necessary for follicular growth and oocyte maturation. Furthermore, both receptors were co-localised in granulosa and theca cells. To date, this has been reported only in the spiny mouse (*Acomys cahirinus*) and the common marmoset (*Callithrix jacchus*; Saunders *et al.* 2000). Both tESR1 and tESR2 were also detected in the

corpus luteum (CL). ER–oestrogen interactions occur in the CL of other species (Telleira *et al.* 1998, Chiang *et al.* 2000), and in the tammar, tESR1 in conjunction with ESR2 may similarly be involved in the steroid secretory function of the CL which is controlled by the LH surge as well as in mediating autocrine and paracrine intraovarian regulation as observed in the human (Misao *et al.* 1999) and mice (Byers *et al.* 1997). Both tESR1 and tESR2 were co-expressed in the granulosa and theca cells of follicles at different stages of development, as seen in the rabbit (Iwai *et al.* 1991) and human (Tetsuka *et al.* 1998) but not in mice and rats. In the rat, ESR1 is localised to theca cells and interstitial tissue while ESR2 is present in granulosa cells (Hurst *et al.* 1995, Couse & Korach 1999, Sar & Welsch 1999, Chiang *et al.* 2000). The overlapping distribution of the ERs in the tammar ovary suggests that they may be similarly regulated.

Effect of exogenous oestrogen on ER expression

ESR1 protein expression in the gonads exposed to exogenous oestrogen was reduced compared to the control testes because they took on an ovarian appearance. However, receptor mRNA was not significantly lower in treated gonads. Numerous studies show that oestrogen and synthetic oestrogens such as diethylstilboestrol downregulate expression of their own receptors, predominantly affecting *ESR1* (Atanassova *et al.* 2001, Couse *et al.* 2001, Miyagawa *et al.* 2004). ER protein half-lives in mammalian cells are regulated in an oestradiol (OE₂)-dependent manner through the ubiquitin–proteasome pathway (Nawaz *et al.* 1999). Therefore, rapid destruction of the protein may explain the less intense staining of tammar gonads cultured with oestrogen compared to the controls. Since there was only a single (terminal) time point used for analysis (after 5 days of culture) when tissues were sampled, we are unable to determine when the degradation and production of new ER protein occurred or its rate of turnover

Figure 7 ESR1 and ESR2 in cultured gonads treated with and without oestradiol from day 25 of gestation. Seminiferous tubules (St) formed in the control testes (a–c). The Sertoli cells (SC) had cytoplasmic staining for ESR1 (a) and ESR2 (b). There was also cytoplasmic staining in the germ cells (G). In the oestrogen-treated gonads (d–f), ESR1 (d) and ESR2 (e) staining was predominantly nuclear in granulosa-like cells and some germ cells (G; arrowheads, enlargements are shown in the bottom left). There were no seminiferous tubules formed in oestradiol cultured gonads. Negative controls (c, f) show no staining when primary antibody is conjugated with blocking peptides for both ESR1 (c) and ESR2 (f).

Figure 8 Quantitative PCR of *ESR1* (α) and *ESR2* (β) in XY gonads from (a) day 25 of gestation and (b) day 0–2 pp cultured with and without oestrogen. *ESR1* and *ESR2* mRNA relative to β -actin (ΔC_t) was not significantly affected by the presence or absence of oestrogen, irrespective of the stage of the gonads at which the cultures commence.

during the period of culturing. The pattern of tESR1 localisation in the oestrogen-cultured XY gonads resembled that of the undifferentiated ovary except for one major characteristic, namely that protein distribution had changed from a primarily cytoplasmic distribution to a primarily nuclear distribution suggesting that the receptors were activated after treatment (Fig. 7d and e). Coveney *et al.* (2001) obtained gonadal sex reversal including germ cell sex reversal, after treatment of neonatal pouch young with OE₂. Clearly, culture of XY gonads with OE₂ also induces a degree of gonadal sex reversal.

The expression of tESR2 in the gonads cultured with oestrogen was similarly not significantly affected. *ESR2* may act antagonistically to regulate *ESR1* in the presence of oestrogen without requiring a high or increased level of expression (Pettersson & Gustafsson 2001, Lindberg *et al.* 2003). Like tESR1, tESR2 protein was reduced and its distribution changed to become predominantly nuclear localisation following oestrogen exposure. The nuclear translocation of both receptor proteins in

the presence of exogenous oestrogen in culture confirms the ability of the receptors to respond functionally to the ligand even at early developmental stages.

Coveney *et al.* (2001) obtained gonadal and germ cell sex reversal after oestrogen treatments of neonatal pouch young for 25 days. In our study, culture of XY gonads with oestrogen also induced morphological sex reversal. We have shown that the receptors can respond to exogenous oestrogen at this early stage and translocate to the nucleus in its presence, but we cannot exclude the potential for non-classical signaling pathways in this process. However, evidence from the ARKO and double-ER (α/β ERKO) knockout mice suggest that the effects of the classical and non-classical pathway are not required for any aspect of early sexual differentiation. Nevertheless, gonadal sex reversal in the presence of oestrogen provides a powerful tool for understanding the interplay of genes and hormones in early gonadal differentiation. We hypothesise that ERs in the indifferent mammalian gonad are capable of transducing the signals from exogenous oestrogen to downregulate the testis differentiation pathway and allow ovarian differentiation to occur. However, the selective forces that maintained conserved ER expression in this tissue remain unknown.

Materials and Methods

Animals

Tammar wallabies from Kangaroo Island (South Australia) were held in open grassy yards in our breeding colony. Natural diet was supplemented with lucerne cubes, oats and fresh vegetables and water *ad libitum*. All experiments were approved by the University of Melbourne Animal Experimentation Ethics Committees, and animal handling and husbandry were in accordance with the National Health and Medical Research Council of Australia (2004) guidelines.

Timed births were obtained after initiating pregnancy by removal of pouch young (RPY), which reactivates the quiescent, diapausing blastocyst during the breeding season (Tyndale-Biscoe & Renfree 1987). The day of RPY is designated day 0 of the 26.5-day pregnancy (Tyndale-Biscoe & Renfree 1987). For foetal tissue collection, adult females were killed by anaesthetic overdose (60 mg/ml sodium pentobarbitone to effect) from day 23 to day 25 of gestation, and the reproductive tract was removed. Foetuses were removed from the tract, and both gonads and mesonephroi were dissected out. From each animal, one gonad was snap frozen for RNA analysis, and the other was fixed in 4% paraformaldehyde (PFA) for histological analysis. A piece of tail was collected from each foetus for sexing by PCR for the presence of *SRY* (Harry *et al.* 1995). Pouch young were removed from mothers (RPY) at d1–13 ($n=5$), 23–24 ($n=2$), 49 ($n=2$), 60 ($n=2$), 72 ($n=2$), 90 ($n=2$), 150 ($n=2$ ovaries) and ($n=2$ testes), and d300 ($n=2$) ovaries. Day 0–20 pouch young were killed by decapitation, and older pouch young were anaesthetised with an i.p. injection of sodium pentobarbitone then decapitated. Adult ovaries ($n=10$) and testes ($n=10$) were collected for

histology and gene expression analysis as previously described (Renfree & Tyndale-Biscoe 1978). Non-reproductive tissues were collected from adult females, including liver ($n=5$), adrenal ($n=5$), pituitary ($n=5$), kidney ($n=5$) and hypothalamus ($n=5$).

Culture of XY gonads with oestrogen

Pairs of gonads with mesonephroi intact were collected from male foetuses ($n=10$) at d25 of gestation. Gonads were cultured according to the method described by Martineau *et al.* (1997). From each pair, one gonad was cultured without oestrogen (control), and the other was cultured with oestrogen. The control medium consisted of DMEM (Thermotrace, Victoria, Australia) supplemented with FCS (+10%) (Thermotrace), penicillin–streptomycin (penicillin-G, 100 IU; streptomycin 100 µg/ml, Thermotrace), insulin–transferrin–selenium (100 µg/ml, Gibco) and L-glutamine (2 mmol/ml, Gibco). The oestrogen medium was the control medium supplemented with oestrogen (0.1 ng/µl). Gonads were cultured in organ dishes (Falcon, York, UK) on fine wire that supported the gonad at the surface of the culture medium. Gonads were cultured for 5 days in a humidified environment at 37 °C in 6% CO₂ in air, and culture media were replenished every second day. After culture, one of each pair of cultured gonads was fixed in 4% PFA for histology, and one was snap frozen for RNA analysis.

RNA extraction and RT-PCR

RNA was extracted from small tissues (<80 mg) using the GenElute Mammalian Total RNA Miniprep Kit (Sigma, #RTN-70) as per the manufacturer's instructions. Tissues weighing more than 100 mg were homogenised in 1 ml RNAwiz (#9736; Ambion, Victoria, Australia). Up to 5 µg of RNA was treated using the Superscript III First Strand Synthesis System for RT-PCR (Invitrogen, #18080-051) to generate first strand cDNA according to the manufacturer's instructions.

PCR

PCR conditions were as follows: initial incubation of 94 °C for 2 min, followed by 36 cycles of 94 °C for 1 min, primer-specific annealing temperature for 1 min, and 72 °C for 1 min, with a final extension at 72 °C for 5 min. Promega Taq DNA polymerase B (Promega, #M1661) and accompanying reagents were used, with 1.5 µM MgCl₂ and 0.2 µM each (dNTPs).

Quantitative PCR

Quantitative real-time PCR (qPCR) using a SYBR green Mastermix (Quantitect; Qiagen) and an Opticon 2 Monitor (Geneworks; MJ Research, Waltham, MA, USA) was used to measure relative *ESR1* and *ESR2* gene expression in XY oestrogen-cultured and control gonads. Specific primers were designed to optimise qPCR sensitivity (Table 2). All qPCR reactions were performed in triplicate using 96-well multiplates and a reaction volume of 20 µl, and both *ESR1* and *ESR2* were measured relative to β -actin. The data were analysed

using the ΔC_t method described by Pfaffl (2001). *P* values were calculated using a standard student's *t*-test. The conditions used for qPCR were as follows: 50 °C for 10 min, 95 °C for 15 min, followed by 39 cycles of 95 °C for 30 s, 59 °C for 20 s, 72 °C for 40 s, 78 °C for 1 s, 72 °C (all genes were optimised to this temperature) for 1 min, and 15 °C until the end of the reaction.

Northern blot

Total RNA was extracted from ovary, testis, kidney, liver, adrenal, pituitary, hypothalamus and mammary gland, for northern analysis of *tESR1* and *tESR2* mRNA expression, according to standard methods (Zeta-Probe GT Genomic Tested Blotting Membrane (Bio-Rad, #162-0196; Megaprime Labelling System, Amersham #RPN1604/5). An *ESR1*-specific cDNA probe of 400 bp and *ESR2*-specific cDNA probe of ~300 bp were used to detect the receptors in the tissues mentioned above. Membranes were exposed to Fuji medical X-ray film (Super RX, #497690; Hanimex, Brookvale, NSW, Australia), and films were developed using an All-pro 100 Plus XOMat machine (#2792; NuvoSolutions/All imagingCorps, Victoria, Australia; see western blot section).

Immunohistochemistry

Tissues were fixed in 4% PFA and stored for 2–3 h at 4 °C. The tissue was then rinsed in PBS three times for 30 min each and finally stored in 70% ethanol (EtOH) at 4 °C. Tissues were sectioned at 6 µm. Polyclonal rabbit antibodies Ab-16 (*ESR1*, Neomarkers #RB-1493-P1) and Ab-24 (*ESR2*, Neomarkers #RB-037-P) were diluted to a final concentration of 1:100, and a biotinylated secondary goat anti-rabbit antibody was used with ABCComplex/HRP kit (DAKO, #K0355) and colour was developed with DAB Chromagen tablets (DAKO, #S3000). Sections were counterstained with haematoxylin. Appropriate negative control reactions were run in parallel (*ESR1* peptide 1:20, Neomarkers, #PP-1493 and *ESR2*, Neomarkers, #PP-037-P). Follicles in ovarian sections were classified as previously described (Tyndale-Biscoe & Renfree 1987, Richings *et al.* 2006).

Western blot analyses

Ovarian tissue was homogenised in a radio-immunoprotein assay buffer (50 mM Tris base, 150 mM NaCl, 1% NP-40, 0.25% Na-deoxycholate) supplemented with protease inhibitors (Protease Inhibitor Cocktail Set V, #535141; 1 µl/ml; Calbiochem, Merck). The protein extract was sonicated, on ice, seven times for periods of 15 s on and 15 s off. Twenty-five micrograms of extracted protein was analysed by SDS-PAGE using the discontinuous buffer system described by Laemmli (1970). Proteins were transferred onto nitrocellulose membrane using a semi-dry trans cell electro-blotter (Tr4ansblot SD cell #22981, Bio-Rad) at 15 V for 15 min. Stored membranes were blocked overnight with 45% fish gelatin in 1×PBS–Tween at 4 °C. Primary antibody was added at a concentration of 1:500 and incubated at room temperature for 1 h. The second antibody was incubated at room temperature at a concentration of 1:1000 for 1 h.

The membrane was exposed to Hybond ECL hyperfilm using Luminol reagents (#sc-2048; Santa Cruz Biotechnology, Santa Cruz, CA, USA) 3 min and developed using an All-pro 100 Plus XOMat machine (NuvoSolutions/All imagingCorps #2792).

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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