Effect of single and compound knockouts of estrogen receptors α (ERα) and β (ERβ) on mouse reproductive phenotypes

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SUMMARY

The functions of estrogen receptors (ERs) in mouse ovary and genital tracts were investigated by generating null mutants for ERα (ERαKO), ERβ (ERβKO) and both ERs (ERαβKO). All ERαKO females are sterile, whereas ERβKO females are either infertile or exhibit variable degrees of subfertility. Mast cells present in adult ERαKO and ERαβKO ovaries could participate in the generation of hemorrhagic cysts. Folliculogenesis proceeds normally up to the large antral stage in both ERαKO and ERβKO adults, whereas large antral follicles of ERαKO/−/ERβKO and ERαβKO adults are markedly deficient in granulosa cells. Similarly, prematurely developed follicles found in prepubertal ERαKO ovaries appear normal, but their ERαβKO counterparts display only few granulosa cell layers. Upon superovulation treatment, all prepubertal ERαKO females form numerous preovulatory follicles of which the vast majority do not ovulate. The same treatment fails to elicit the formation of preovulatory follicles in half of the ERβKO mice and in all ERαKO/−/ERβKO mice. These and other results reveal a functional redundancy between ERα and ERβ for ovarian folliculogenesis, and strongly suggest that (1) ERβ plays an important role in mediating the stimulatory effects of estrogens on granulosa cell proliferation, (2) ERα is not required for follicle growth under wild type conditions, while it is indispensable for ovulation, and (3) ERα is also necessary for interstitial glandular cell development. Our data also indicate that ERβ exerts some function in ERαKO uterus and vagina. ERαβKO granulosa cells localized within degenerating follicles transform into cells displaying junctions that are unique to testicular Sertoli cells. From the distribution pattern of anti-Müllerian hormone (AMH) in ERαβKO ovaries, it is unlikely that an elevated AMH level is the cause of Sertoli cell differentiation. Our results also show that cell proliferation in the prostate and urinary bladder of old ERβKO and ERαβKO males is apparently normal.

Key words: Knockout mice, Functional redundancy, Ovary, Uterus, Prostate, Anti-Müllerian hormone, Sertoli cells, Mast cells, Estrogen receptors

INTRODUCTION

Estrogens regulate a wide variety of physiological processes in classical targets as the skeletal and cardiovascular systems (for review and references see Couse and Korach, 1999). Two estrogen receptors (ERs) are known to transduce estrogenic signals: ERα (Green et al., 1986) and ERβ (Kuiper et al., 1996; Mosselman et al., 1996; Tremblay et al., 1997). The domain organization of ERα (Krust et al., 1986; Gronemeyer and Laudet, 1995) is conserved in ERβ (Muramatsu and Inoue, 2000, and references therein). In both ERα and ERβ, the nonconserved N-terminal A/B domain harbors a ligand-independent transactivation function AF1, that can be activated through mitogen-activated protein kinase-mediated phosphorylation of serine residues (Kato et al., 1995; Tremblay et al., 1997). The highly conserved C domain (the DNA-binding domain, DBD) of ERα and ERβ is responsible for their specific binding to estrogen response elements of target genes. The less conserved E domain that contains the ligand-binding domain (LBD), also contributes to receptor dimerization and harbors the AF2 ligand-dependent transactivation function (for Refs see Couse and Korach, 1999).

ERα and ERβ exhibit both differential and overlapping tissue distribution, and test-tube studies, as well as in vitro studies with transfected cultured cells, have revealed overlap, but also significant differences in their ligand-binding and transcriptional properties (for references see Couse and Korach, 1999; Saville et al., 2000). For example, the cell type- and promoter-dependent agonistic activity of 4-hydroxytamoxifen appears to be unique to ERα (Berry et al., 1990; Tremblay et al., 1997), whereas anti-estrogens that block the stimulatory activity of ERα/AP1 complexes appear to be potent agonists when bound to ERβ/AP1 complexes (Paech et al., 1997). Similarly, transactivation by ERα/Sp1 and ERβ/Sp1 complexes through Sp1 promoter elements is cell type-, ligand- and promoter-dependent (Saville et al., 2000).

Both ERα (Lubahn et al., 1993) and ERβ (Krege et al., 1998)
Genes have been previously disrupted by targeted mutagenesis (αERKO and βERKO mutant mice, see Couse and Korach, 1999). Both disruptions lead to pleiotropic effects, but the αERKO and βERKO phenotypes are mostly distinct. It is, however, unclear whether the mutation generated by disruption of the gene for Erα in αERKO mice actually represents a null mutation, as a transcriptionally active form of Erα truncated for the A/B domain is present in low amounts in these mice (Couse et al., 1995). Because we wish to characterize in vivo the individual role of the AF1 and AF2 activation functions of Erα and Erβ, with respect to their estrogen- and anti-estrogen dependence, their target genes, their phosphorylation and their function in the stimulatory activity of ER/AF1 and ER/AF2 complexes (see above), we have decided to generate new Erα- and Erβ-null mutant mice, that would serve as references for future genetic dissection of Erα and Erβ functional domains. We report here the generation of mice that fully lack Erα (ErαKO mutants), Erβ (ErβKO mutants), and both Erα and Erβ (ErαβKO mutants), and the comparison of their reproductive tract and gonad phenotypes with those of the previous αERKO, βERKO and αβERKO (Couse et al., 1999b) mutants.

**MATERIALS AND METHODS**

**Generation of Erα null mutant mice (ERαKO)**

Mouse Erα (mErα) genomic clones were obtained by screening a 129/Sv embryonic stem (ES) cell DNA library, with a mErα CDNA probe (nucleotide 177 to 2007; White et al., 1987). A targeting vector was generated from a 9 kb BamHI fragment containing exon 3 (nucleotide 655 to 845, amino acids 156–218; White et al., 1987) encoding the first zinc finger of the DBD (Fig. 1a). The TKneo cassette from pHR56 (Metzger et al., 1995) was cloned into the Eco47III site, and a loxp site followed by a KpnI site were introduced at the NheI site by PCR-based site-directed mutagenesis (Fig. 1a). The 10 kb Pml-Hpal fragment of the targeting vector (Fig. 1a) was electroporated into 129/SvPas H1 ES cells (Dierich and Döll, 1997) and G418 neomycin-resistant clones were expanded (Lufkin et al., 1991).

ES cells containing a targeted ErαL3 allele were identified by Southern blot analysis of BamHI-digested ES cell genomic DNA, using 5’ (P5) and 3’ (P3) external probes and a ‘neo’ probe (Fig. 1a). Targeted ES cells were injected into C57BL/6 blastocysts and returned to a pseudopregnant host of the same strain. Chimeric males were obtained that transmitted the mutation through crosses with C57BL/6 heterozygotes. Total RNA was extracted from uterus (Erα) or ovaries (Erβ) described (Auffray and Rougeon,1980). Reverse transcription was carried out on 1 μg of total RNA with M-MuLV reverse transcriptase (Bios). Subsequent nested PCR was performed to amplify full-length coding cDNAs of Erα (1.8 kb) and Erβ (1.65 kb). In the case of Erα, two sets of primers (coordinates as in White et al., 1987) were used: set 1 primers (30 cycles), A 5’-CCGGTCGACCATCATATCACCCGCTTTCTCA-3’ and B 5’-GACGAGCTGCACCACTGTCGCAGT-3’ (nucleotides 1886-1900); set 2 primers (30 or 45 cycles), B 5’-AGATTCATAGCTGATGGGACGCT-3’ and A 5’-AGGGAGCCGTTGGAGTTCATCAG-3’ (nucleotides 188-194) and A’ 5’-GGGGAGCCGTTGGAGTTCATCAG-3’ (nucleotides 1973-1994). In the case of Erβ, set 1 primers, A 5’-TCTCTAGAGGTCATCTGTCGCAGT-3’ and B 5’-CGAGCTCCGACCATATCGATC-3’ (nucleotides 239-411) were used. The Erβ amplified products were analyzed by Southern blotting with four 5’-radiolabeled oligonucleotides specific for Erβ exons 3, 4, 5 and 6, and by sequencing.

**ERα western blotting**

Protein extracts were prepared from WT and ErαKO uterus according to Rochette-Egly et al. (1994). 50 μg of protein were separated on 10% gel by SDS-PAGE and transferred onto nitrocellulose membrane. Erα was detected with a rabbit polyclonal antibody (MC20, Santa Cruz Biotechnology). The same blot was hybridized with an anti-actin antibody used as internal control.

**Immunohistochemistry**

Immunoperoxidase labeling for detection of Erβ was performed on frozen sections of ovariates, postfixed in Zamboni’s fluid, using a polyclonal antibody raised against a peptide corresponding to residues 467–485 (Tremblay et al., 1997) of the Erβ region F. Immunoperoxidase labeling for detection of AMH was carried out on histological sections from ovariates fixed in Bouin’s fluid and embedded in paraffin (Al-Attar et al., 1997). The sections were counterstained with Harris’ Haematoxylin. Cell proliferation was assessed by immunoperoxidase labeling on histological sections from paraformaldehyde-fixed, paraffin-embedded prostates using the antibody NCL-Ki67p (Novocastra Laboratories). Peroxidase activity was revealed using 4-chloro-1-naphthol (Merck) as a substrate (nuclei counterstained with DAPI).
Bromodeoxyuridine (BrdU) incorporation into DNA
12- and 20-month-old ERβKO and control males (WT and ERβ+/−) as well as 8-month-old ERαKO and control males (ERβ+/− and ERα−/+), were injected intra-peritoneally (IP) four times every 2 hours with 10 mg BrdU/kg of body weight and sacrificed 2 hours after the last injection. Prostates were fixed in Bouin’s fluid for 16 hours at 4°C, embedded in paraffin and BrdU incorporation revealed using an anti-BrdU mouse monoclonal antibody (Boehringer Mannheim) and immunoperoxidase labeling (nuclei counterstained with Haematoxylin). BrdU-labeled nuclei were quantitated by counting at least 2000 nuclei.

Fertility of ER mutant mice
Young females (7-15 weeks old) and fertile males were bred during 15 weeks. The presence of seminal plugs, the number of pups per litter and the number of litters per female were scored. Male fertility was similarly tested by breeding young males and fertile females.

Superovulation and oocyte quantification
21- to 25-day-old wild-type and ER mutant females were injected IP with 10 units of PMSG (Folligon® -Intervet) followed by 5 units of hCG. Progeny were counted after enzymatic dissociation from the surrounding cumulus with hyaluronidase (37°C, 1 hour; Hogan et al., 1994). Oocyte/cumulus masses were extracted from oviducts, and oocytes later, and sacrificed 19-22 hours after the hCG injection.

RESULTS
Generation of ERα and ERβ single and double null mutant mice (ERαKO, ERβKO and ERαβKO)
We targeted exon 3 that encodes the first zinc finger of the DBD to generate a fully disrupted ERα mutant. The homologous recombination targeting vector (Fig. 1a) was designed with three loxP sites flanking exon 3 and the TKneo cassette (see Fig. 1a, and Materials and Methods). Complete Cre recombinase-mediated excision of exon 3 and ‘floxed’ cassette in the L3 allele (Fig. 1a) should result in the creation of a stop codon at the new amino acid position 158 generated by splicing exon 2 and 4 transcripts (White et al., 1987; Couse et al., 1995; A. K., unpublished). Thus, the putative truncated protein produced from the deleted allele (L-) would be 157 amino acids long, lacking the C to F regions of ERα. However, partial Cre-mediated excision of the ‘floxed’ selection cassette should yield the conditional ‘floxed’ allele L2 (Fig. 1a).

Chimeric males derived from targeted L3 ES clones transmitted the mutation through their germline. Inbreeding of heterozygous ERα−Δ−/− mice (see Materials and Methods) yielded ERαΔ−Δ− mice in accordance with Mendelian expectation. The full disruption of the gene for ERα was confirmed by the absence in ERαKO uterus of any ERα polypeptide immunoreacting with an antibody directed against the F region of ERα (Fig. 1b). Moreover, no ERαRNA containing transcripts of any of the exons located downstream of exon 3 could be detected by RT-PCR (Fig. 1c, and data not shown). Collectively, these results demonstrate that the ERαΔ−Δ− mice are indeed ERα null mutants that we also designated as ERα−/− or ERαKO mutant. Note that in the previously generated mouse αERKO mutant, ERα was disrupted by inserting a neo gene into exon 2 (Lubahn et al., 1993). No full-length ER mRNA could be detected, but one smaller transcript variant encoded a truncated ERα protein still possessing the DBD and the LBD of wild-type (WT) ERα, as well as significant estrogen-dependent transcriptional capacity (see Couse et al., 1995).

The mouse gene for ERβ was disrupted through homologous recombination by inserting the neo gene into the Spel site of exon 3, which encodes the first zinc finger of the DBD (Fig. 1d, and Materials and Methods). Chimeric males derived from targeted ES cell clones transmitted the mutation through crosses with C57BL/6 females. Inbreeding of heterozygote mice yielded a Mendelian distribution of all expected genotypes. To establish that ERβ was actually lacking in these mutants, we first examined ERβ transcripts using RT-PCR performed on total RNAs extracted from ERβ+/+ and ERβ−/− adult mouse ovari(es (Fig. 1e; see Materials and Methods). The expected 1.6 kb cDNA species was obtained with WT RNAs, but not with ERβ−/− RNAs, which yielded three smaller cDNA species (ERβ−/− 1 to 3, Fig. 1e). Their analysis showed that they represent different alternative splicings of ERβ transcripts (Fig. 1e). All three lacked exon 3, thus leading through splicing of exon 2 and 4 transcripts to a frameshift with the creation of two stop codons at the beginning of exon 4 (nucleotide position 416 to 421 in Tremblay et al., 1997) and resulting in a putative 77 amino acid long peptide. Only the targeted exon 3 transcript was spliced out in ERβ−/−1, whereas the transcript of two additional exons (6 and 7) were spliced out in ERβ−/−3. This alternative splicing of both (exons 6 and 7) transcripts has been described in normal mouse tissues (Lu et al., 1998). ERβ−/−2 was not fully characterized, but must correspond to an additional splicing occurring downstream of exon 6. Note that in the previously reported similar, but not identical, disruption of the gene for mouse ERβ (βERKO; Krege et al., 1998), a PGK neo gene was inserted in the reverse orientation into exon 3 Plsp site (nucleotides 329-334, Tremblay et al., 1997), i.e., downstream of our insertion site, into the sequence encoding the first zinc finger of the DBD. Several similar, but not identical splicing variants were also found in this βERKO mutant, in which the exon 3 transcript was consistently spliced out. In two described transcripts, this splicing out generated the same frameshift and stop codons as in our ERβKO mutant, and resulted in the same putative truncated peptide that lacks both DBD and LBD. However, in the third described transcript, exon 3 and exon 4 splicing out resulted in an open reading frame yielding a putative polypeptide lacking the DBD.

Our disruption of the gene for mouse ERβ was also controlled by immunohistochemistry (Fig. 1f). No immunostaining could be detected on ERβ−/− ovary sections, while a clear signal was observed in WT granulosa cell nuclei. Thus, the above data indicate that our present ERβ disruption results in a null mutant (ERβ−/− or ERβKO) that is most probably functionally identical to the previous βERKO mutant (Krege et al., 1998).

ER double heterozygous mice (ERα−Δ−/−/ERβ−/−), obtained from breeding ERα−Δ−/− females with ERβ−/− males, were inbred to generate mutant mice homozygous for ERα and ERβ disruption, designated as ERαβKO. All genotypes were obtained in agreement to Mendelian expectation and with a normal sex ratio (data not shown).

Fertility of ERα and ERβ single and compound mutants
In continuous mating studies, our ERαKO and ERαβKO males
were infertile (data not shown) in accordance with previous αERKO (Eddy et al., 1996) and αβERKO (Couse et al., 1999b) data. However, as reported for βERKO males (Krege et al., 1998), our ERβKO, as well as ERα+/−/ERβKO males, exhibited normal fertility (data not shown).

Continuous mating studies were also carried out in females.
to investigate the reproductive consequences of ERα and ERβ mutations. In accordance with previous αERKO data (Lubahn et al., 1993), our ERαKO females were infertile as shown by the total absence of copulatory plugs and pregnancy (Table 1). In contrast, ERβKO females were either infertile or exhibited a reduced fertility, even though they presented vaginal plugs, indicating a normal sexual behaviour (data not shown). An analysis of 12 ERβKO females showed that three groups could be distinguished. Six females (A) were subfertile with a normal number of litters (3.17±0.17) and a reduced number of pups per litter (3.95±0.34), while three others (B) had a much more reduced fertility (one litter and 2.00±0.58 pups per litter) and the remaining three (C) were apparently infertile. Interestingly, disruption of one ERα allele in the ERβKO null background (ERα+/−/ERβKO mutants) resulted in complete female infertility.

Table 1. ER mutant female fertility in continuous matings

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Litters</th>
<th>Pups per litter</th>
<th>Pups per female</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT, ERβ+/−</td>
<td>22</td>
<td>68</td>
<td>462</td>
<td>6.79±0.31</td>
</tr>
<tr>
<td>ERαKO</td>
<td>6</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ERβKO (A)</td>
<td>6</td>
<td>19</td>
<td>75</td>
<td>3.95±0.34*</td>
</tr>
<tr>
<td>ERβKO (B)</td>
<td>3</td>
<td>3</td>
<td>6</td>
<td>2.00±0.58‡</td>
</tr>
<tr>
<td>ERβKO (C)</td>
<td>3</td>
<td>0</td>
<td>0</td>
<td>1.00±0.00§</td>
</tr>
<tr>
<td>ERα+/−/ERβKO</td>
<td>7</td>
<td>0</td>
<td>0</td>
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</tbody>
</table>

Control (WT, ERβ+/−) and mutant females (7-15-week-old) were bred with fertile males for 15 weeks. The number of pups per litter and the number of litters per female were scored. ERβKO females responding differently are separated in three groups (A), (B) and (C). Results are presented as mean±s.e.m.

*Statistically different from control females, P=0.0001 (unpaired t-test).
‡, §Statistically different from ERβKO (A) females.
‡ P=0.041 (unpaired t-test).
§ P=0.0001 (unpaired t-test).

Genital tract phenotype of adult ERαβKO females

For each mutant genotype (ERαKO, ERβKO and ERαβKO), as well as for wild type (WT) age-matched ‘controls’, ovaries and genital tracts from at least three mice were analysed. The genital tracts of sexually mature ERβKO and ERα+/−/ERβKO mice were similar to those of WT females. In contrast, ERαKO uterus and vagina were hypoplastic and did not display cyclic changes (Fig. 2a-c, e.g. and data not shown). In ERαβKO females, the lengths of the oviducts, uterine horns and vagina were normal, indicating that signaling through ERs is not required for the growth of the genital tract along the antero-posterior body axis (Fig. 2a, b). However, the diameter and wall thickness of the ERαβKO uterus and vagina were, on average, reduced twofold when compared with their ERαKO counterparts (Fig. 2a-h). The increased uterine and vaginal hypoplasia generated upon inactivation of the gene for ERβ in the ERαKO genetic background was equally distributed between epithelium, lamina propria and smooth muscles, and reflected decreases in both cell number and size (compare E, LP and M in Fig. 2e-h).

Structural analysis of adult ovaries of ERα and ERβ single and compound mutants

Ovaries of WT adult mice (OV, Fig. 2b) consist of a follicular and an interstitial compartments. The follicular compartment comprises primordial (PR, Fig. 3a), growing (e.g. A, Fig. 3a and c) and atretic follicles (not shown), as well as corpora lutea (CL, Fig. 3a). The interstitial compartment appears to be formed by small cells, which do not contain granules (Fig. 3a). The interstitial compartment contains small cells, which do not contain granules (Fig. 3a).
compartment (IT, Fig. 3a) is most prominent in the ovarian medulla and consists of compact clusters of large glandular cells (IG, Fig. 3b), numerous fibroblasts, capillaries (CP, Fig. 3b) and rare mast cells (not shown). Adult ERβKO ovaries were macroscopically normal and showed normal antral follicles, but in most of them corpora lutea were scarce or absent (not shown). In contrast to Krege et al. (1998), we did not detect any increase in the number of atretic follicles in our ERβKO ovaries.

Ovaries from adult ERαKO mice (14-week- or 6-month-old) displayed (1) large, frequently hemorrhagic, cysts (Fig. 2b) originating from antral follicles (CY, Fig. 3d); (2) normal primordial, primary and antral follicles (e.g. P and A, Fig. 3d and f); (3) a large excess of follicles at advanced stages of atresia (D, Fig. 3d); (4) an absence of typical mature corpora lutea (Fig. 3d, and data not shown); and (5) a marked reduction in the number of glandular interstitial cells that appeared loosely arranged as a consequence of edema of the interstitial tissue (IG and asterisks in Fig. 3e). Moreover, numerous deep-blue-stained cells were scattered in the interstitial compartment of ERαKO ovaries (M, Fig. 3e), which contained dozens of granules staining metachromatically with toluidine blue (Fig. 3e, inset). These features are characteristic of mast cells (Fawcett, 1986).
A polycystic ovarian phenotype similar to that of ERαKO mice was observed in adult (14-week- and 6-month-old) ERαβKO mice (OV, Fig. 2b; CY, Fig. 3g, and data not shown). Components of the follicular compartment (including primordial, primary, small antral, degenerating and hemorrhagic cysts (CY and D, Fig. 3g, and data not shown)) and cellular composition of the interstitial compartment (e.g. M, Fig. 3h, and data not shown) were similar in ERαβKO and ERαKO ovaries. However, the largest antral follicles of ERαβKO ovaries were abnormal in that (1) their walls were locally very thin, owing to a lack of granulosa cells (A, Fig. 3g,i, and compare the green brackets in Fig. 3c,f,i); and (2) they never reached the size of their counterparts in WT and ERαKO ovaries (Fig. 3c,f,i).

Interestingly, disruption of only one allele of ERα from the ERβ null genetic background (ERα+/−/ERβKO) resulted in ovaries that consisted of corpora lutea and displayed numerous abnormal antral follicles with similar characteristics to those of ERαβKO ovaries. However, ERα+/−/ERβKO ovaries displayed no hemorrhagic cysts and showed a normal organization of interstitial glandular cells with rare mast cells (data not shown).

Strikingly, all ERαβKO ovaries, but none of the ERαKO and ERβKO single mutant ovaries, contained unusually large cells that exhibited an abundant cytoplasm, a pale ‘dusty’ chromatin and a prominent nucleolus and were organized into either irregular clusters (C, Fig. 3g,h; Fig. 3k) or short tubules (T, Fig. 3g,h; Fig. 3j). These cells, which were essentially observed in the ovarian medulla (although few of them were also detected in the ovarian cortex in association with follicles at advanced stages of atresia (D, Fig. 3g)), have no counterparts in normal mammalian ovaries (Harrison and Weir, 1977; Mossman and Duke, 1973). Based on morphological criteria described below, they were identified as fully differentiated Sertoli cells.

Presence of Sertoli cells in adult ERαβKO ovaries
Sertoli cells, which, under normal conditions, are found exclusively in testis seminiferous tubules (Figs 3l,m and 4b,e,h,i), are columnar cells attached to a basement membrane (BM, Fig. 3l,m) whose apaxes possess numerous veil-like processes (AP, Fig. 3l,m) and whose nuclei are ovoid and located near the basement membrane (NU, Fig. 3l,m). These histological features are particularly conspicuous in seminiferous tubules depleted from their germ cells, such as those from vitamin A-deficient (VAD) mice (Ghyselinck et al., 1999; Fig. 3l,m).

When examined by high-resolution light microscopy and electron microscopy, Sertoli cells display characteristic nuclear profiles: the nuclear envelope exhibits deep and narrow indentations (not shown); and the prominent reticular nucleus (N, Figs 3l,m and 4b) is characterized as a large clumps of heterochromatin (or nucleolar satellites, small arrows in Figs 3m and 4b; Kuhn and Therman, 1988). Plasma membrane-cytoskeleton-endoplasmic reticular complex, known as ectoplasmic specializations, are unique to pubertal and postpubertal Sertoli cells. These are formed by regularly spaced belts of circumferential actin filaments (A, Fig. 4e,h,i) that are bordered on the cytoplasmic side by cisternae of endoplasmic reticum (ER, Fig. 4e,h,i) and are surrounding a series of occluding junctions (J, Fig. 4e,h) connecting adjacent Sertoli cells (S1 and S2, Fig. 4h) (Pelletier and Byers, 1992; Weber et al., 1988; Byers et al., 1993). It is noteworthy that in both WT and ERαβKO ovaries, granulosa cells (G, Fig. 4d) are connected via gap junctions (GAP) and desmosomes (D), but not by occluding junctions, although they represent the female homologs of Sertoli cells, with respect to their embryological origin from the genital ridges (Swain and Lovell-Badge, 1999), their function of nurse cell for the germ-cell lineage and their gene expression pattern (see for example Couse et al., 1999b and references therein).

The abnormal cells found in the ovary of adult ERαβKO mutants were unambiguously identified as Sertoli cells based on the following criteria: (1) they were connected by typical ectoplasmic specializations indistinguishable from those observed in 2-week-old normal prepubertal testes (compare ER, A and J in Fig. 4c,f,g with 4e,h,i); (2) they displayed satellites flanking a large reticular nucleolus (N and small arrows in Figs 3j,k and 4a), as well as elongated and indented nuclear profiles (arrowhead in Fig. 4a); and (3) their spatial organization within ovarian tubules faithfully mimicked that of authentic Sertoli cells within germ-cell-free seminiferous tubules (compare Fig. 3j with 3l).

Structural analysis of ovaries from prepubertal and young pubertal mice
The follicular compartment of prepubertal, 23-day-old WT, ERβKO and ERα+/−/ERβKO females contained only rare small antral follicles (A, Fig. 5a,c; SA, Fig. 6a; and data not shown). In contrast, some large antral follicles of normal structure were seen in prepubertal ERαKO ovaries (LA in Fig. 6b) and the vast majority of the growing follicles in prepubertal ERαβKO ovaries possessed a well-defined antrum (A compare Fig. 5a with 5b; SA, compare Fig. 6a with 6c). The structure of the ERαβKO antral follicles was abnormal as: (1) the granulosa cells either formed a single layer (black arrowheads in Fig. 5b,d) or appeared loosely organized owing to the presence of multiple small cavities filled with follicular fluid (Fig. 5b,d); (2) the theca cells always formed a single layer (versus 2 to 4 layers in WT, ERαKO and ERβKO follicles; compare green brackets in Fig. 5c,d and data not shown); and (3) in a majority of the follicles, the oocyte was completely separated from the follicular wall and floated in the follicular fluid (O, Fig. 5b,d).

Altogether, these results strongly suggest that the secretory activity of the follicles increases prior to the normal onset of puberty in both ERαKO and ERαβKO mice. Additionally, in ERαβKO ovaries the proliferation rate of theca cells was apparently diminished.

Sertoli-like cells were not detected in prepubertal (i.e., 23-day-old) ERαβKO ovaries. However, all ERαβKO ovaries at the onset of puberty (i.e., 30 days) displayed, essentially between the follicles, but also within some atretic follicles, clusters of densely packed cells with abundant cytoplasm, pale elongated nuclei and large reticular nucleoli (S, Fig. 5f,h,i,j), that had no counterparts in age-matched WT, ERαKO and ERβKO ovaries (e.g., Fig. 5e,g). These cells lacked indentations of the nuclear envelope and nucleolar satellites (Fig. 5j), but were connected by ectoplasmic specializations (Ac, ER and J, Fig. 5k) and thus resembled normal immature Sertoli cells (Byers et al., 1993). With few exceptions (e.g. Fig. 5f,h), the clusters of Sertoli cells located within the atretic
ovarian follicles of peripubertal ERαβKO mice were small (two to five cells on average; Fig. 5i,j). The ectoplasmic specializations between extrafollicular Sertoli cells were identical to those observed in adult ovaries (see above), whereas those of intrafollicular Sertoli cells were less mature (Ac, ER and J in Fig. 5k). Therefore, although the vast majority of the Sertoli cells present in the ovaries of early post-pubertal and adult of ERαβKO mice are extrafollicular, they appear to arise, at least in part, within the epithelium of atretic follicles.

**Expression of anti-Müllerian hormone (AMH) in ERαβKO ovaries**

AMH expression is specific to Sertoli cells of the immature testis and granulosa cells of the adult ovary (Hirobe et al., 1992; Münsterberg and Lovell-Badge, 1991; and references therein), but its ectopic expression can induce the formation of seminiferous tubules in fetal ovaries (Vigier et al., 1987; Behringer et al., 1990). We used immunohistochemistry to investigate the possibility that overexpression of AMH in ERαβKO ovaries might promote the differentiation of Sertoli cells. In adult (i.e., 14-week-old) and early post-pubertal (i.e., 30-day-old) ERαβKO ovaries, AMH was detected in primary and in small antral follicles (P and SA, Fig. 7b-d) and was absent from granulosa cells of large antral and atretic follicles (LA and D, Fig. 7b,d,e). This distribution pattern of AMH was indistinguishable from that observed in age-matched WT ovaries (Ueno et al., 1989; Baarends et al., 1995; Fig. 7a, and data not shown). Moreover, follicles showing synthesis of

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**Fig. 4.** Electron microscopic comparison of Sertoli-like cells in ERαβKO (ERαβ) ovaries, Sertoli cells in WT testes, and granulosa cells in ERαβKO ovaries, as indicated. (a-g) are from 6-month-old mice and (h,i) from a prepubertal male. Note that (f,h) are sections through adjacent Sertoli cells (S1 and S2), while (g) and (i) are sections through the cytoplasmic side of ectoplasmic specializations. Small arrows in panels (a) and (b) point to nucleolar satellites and the arrowhead to an indentation of the nuclear envelope. A, bundles of actin microfilaments; D, desmosomes; ER, cisternae of endoplasmic reticulum at sites of ectoplasmic specializations; F, fibroblastic cell; G, granulosa cell; GAP, gap junction; J, occluding junctions; M, mitochondria; N, nuclei; NU, nuclei; S, Sertoli cell; SP, spermatocytes. Scale bar: 4 μm in a,b; 0.5 μm in c-e; 0.25 μm in f-i.
Reproductive phenotypes in ER null mutants

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AMH in adult ERαβKO ovaries were conspicuously fewer than in WT ovaries, an observation that is in keeping with a marked increase in number of atretic follicles in these mutant ovaries.

In the normal male, expression of AMH, one of the earliest markers of Sertoli cells, is high in the fetus then decreases progressively after birth to reach background levels prior to the completion of puberty (Münsterberg and Lovell-Badge, 1991). In ERαβKO mice, immunostaining for AMH of ovarian Sertoli cells, which was only faint in post-pubertal ovaries (S, Fig. 7e) and undetectable in adult ovaries (T and C, Fig. 7c), does not appear to recapitulate that observed during normal Sertoli cell differentiation.

Ovarian response of ERαKO, ERβKO and ERαβKO compound mutants to exogenous gonadotropins

To gain further insight into the infertility or reduced fertility, as well as in defects of follicle maturation observed in the ER mutant mice, 21-25-day-old prepubertal mutant and WT mice were treated with gonadotropins to stimulate ovulation. The average yield of oocyte per female was similar in WT,

Table 2. Oocytes produced after superovulation of WT, ERβ+/- and ERα+/-/ERβ+/- females (used as controls), and ERαKO, ERβKO, ERα+/-/ERβKO and ERαβKO mutant females (21-25 days old)

<table>
<thead>
<tr>
<th>Genotype</th>
<th>n</th>
<th>Average</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>9</td>
<td>37.00±10.39</td>
<td>0-87</td>
</tr>
<tr>
<td>ERβ+/-</td>
<td>14</td>
<td>28.79±6.34</td>
<td>1-80</td>
</tr>
<tr>
<td>ERα+/-/ERβ+/-</td>
<td>8</td>
<td>45.75±6.68</td>
<td>21-76</td>
</tr>
<tr>
<td>ERαKO</td>
<td>3</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>ERαKO/ERβ+/-</td>
<td>4</td>
<td>1.00</td>
<td>0-4</td>
</tr>
<tr>
<td>ERβKO (A)</td>
<td>13</td>
<td>17.62±3.59*</td>
<td>4-48</td>
</tr>
<tr>
<td>ERβKO (B)</td>
<td>16</td>
<td>0</td>
<td>–</td>
</tr>
<tr>
<td>ERα+/-/ERβKO</td>
<td>11</td>
<td>0.18</td>
<td>0-2</td>
</tr>
<tr>
<td>ERαβKO</td>
<td>3</td>
<td>0</td>
<td>–</td>
</tr>
</tbody>
</table>

The response of WT, ERβ+/- and ERα+/-/ERβ+/- females are not statistically different. Two types of response were observed for ERβKO females: (A) responded to superovulation, whereas group (B) had no ovulation. The number of oocytes is presented as means±s.e.m.

*Statistically different from control females (P=0.019; unpaired t-test).
ERβ+/− and ERα+/−/ERβ+/− mutants (Table 2). In contrast, superovulation was drastically reduced in ERαKO and ERαKO/ERβ+/− females, as only one of these seven females produced four oocytes, while the others (86%) did not appear to ovulate at all (Table 2, and data not shown). This is at variance with the data obtained with αERKO mutants in which up to 83% of the ERα females superovulated, albeit with a reduced oocyte yield (Couse et al., 1999a; Rosenfeld et al., 2000).

Interestingly, hormonally treated ERβKO females responded according to two modes. Approximately half of them did not yield detectable ova, while the others exhibited a reduced yield of oocytes per female (Table 2). This is also at variance with the previous data of Kreege et al. (1998) who reported that more than 80% of their βERKO females superovulated, albeit with reduced oocyte yields. Strikingly, the introduction of one disrupted ERα allele into the ERβKO background (ERα+/−/ERβKO mutants) fully abrogated the variability in penetrance observed for the superovulation defect in ERβKO mice. Finally, as expected, no ova could be collected from ‘superovulated’ ERαKO females.

As expected, superovulation treatment of WT prepubertal females induced the formation of multiple corpora lutea (CL, compare Fig. 6a with 6d). Ovaries from superovulated ERαKO and ERαKO/ERβ−/− appeared almost filled with healthy unruptured preovulatory follicles (OV, Fig. 6f,i, and data not shown), defined by their broad antrum (A), separation of the cumulus oophorus (CU) from the rest of the follicular epithelium and loose connection of the cumulus oophorus with the oocyte (Parkes, 1960). Similar preovulatory follicles were observed in ovaries of WT mice sacrificed shortly (i.e., seven hours) after hCG injection (Fig. 6h). Ovaries from the ERβKO and ERα+/−/ERβKO mice that failed to ovulate in response to exogenous gonadotropins (Fig. 6g,j; and data not shown) contained numerous large follicles whose antrum were, however, conspicuously less developed than that of preovulatory follicles, and which had not achieved their maturation as their oocyte remained attached to the follicular wall via the cumulus oophorus (Fig. 6j, compare with Fig. 6h,i; and data not shown). Superovulation treatment of ERαβKO mice induced the growth of the antral follicles (compare SA and LA in Fig. 6c and 6e), but never up to the preovulatory stage (compare Fig. 6e with 6f).

Luteal cells were not observed in ERαKO, ERαKO/ERβ−/−, ERα+/−/ERβKO and ERαβKO ovaries, nor in those of ERβKO mice with anovulation. Therefore, prepubertal WT and ERαKO ovaries respond to stimulation by exogenous gonadotropins by the production of preovulatory follicles, whereas, upon similar stimulation, complete follicular maturation was not achieved in some ERβKO and in all ERα+/−/ERβKO and ERαβKO ovaries.

Testis and male urogenital tract in ERαβKO mutants

In agreement with previously published results (Eddy et al., 1996; Hess et al., 1997; Couse et al., 1999b), the testis of our ERαKO and ERαβKO adult males showed a loss of germ cells in the seminiferous tubules, and a marked dilation of straight...
Partial functional redundancy between ER\(\beta\) and ER\(\alpha\) in ovarian follicle growth, ovulation and interstitial cell development.

The actions of estrogen on ovarian follicle growth are not fully understood (Couse and Korach, 1999; Rosenfeld et al., 2000; and references therein). Studies of hypophysectomized rats and mutant mice lacking follicle-stimulating hormone (FSH) or its receptor (FSHR) have shown that early stages of folliculogenesis are independent on both steroids and gonadotropins, whereas estradiol and FSH exert synergistic stimulatory effects on the proliferation of granulosa cells from preantral follicles (Dierich et al., 1998; reviewed in Robker and Richards, 1998b). The arrest in follicle growth after the beginning of antrum formation in ER\(\beta\)KO mice (present report) shows, at the very least, that signaling through ERs is necessary to complete folliculogenesis.

In WT rodent ovary, ER\(\beta\) is the predominant estrogen receptor expressed in granulosa cells (Byers et al., 1997; Sar and Welsch, 1999; Schomberg et al., 1999). However, granulosa cells appear normal in ER\(\beta\)KO mice, and the rarity or absence of corpora lutea in the ovaries of most adult ER\(\beta\) null mutant mice (Krege et al., 1998, and present results) represents the only evidence that folliculogenesis might be impaired. Healthy large antral follicles are present in the ovaries of ER\(\alpha\)KO adults, whereas ovarian follicles at the large antral stage exhibit a conspicuous deficiency in granulosa cells in ER\(\alpha^{+/-}\)/ER\(\beta\)KO and ER\(\alpha\)KO adults. Interestingly, a similar phenotype is observed in follicles that are deficient in cyclin D2, an FSH-responsive gene which is involved in granulosa cell proliferation (Sicinski et al., 1996). Altogether, these data indicate that (1) signaling through ERs is required for the proliferation of granulosa cells, (2) ER\(\alpha\) is mostly dispensable for folliculogenesis, whereas (3) the apparent dispensability of ER\(\beta\) in this process likely reflects a functional compensation of ER\(\beta\) inactivation by ER\(\alpha\) (functional redundancy).

As transgenic overexpression of luteinizing hormone (LH) causes premature formation of antral follicles, the known elevation of endogenous LH levels before the expected time of puberty in ER\(\alpha\) knockout mutants (Couse et al., 1999a), and most probably in ER\(\alpha\)/ER\(\beta\) double knockout mutants, suffices to account for precocious antrum formation in their ovaries. Aside from promoting antrum formation, excess LH increases serum estradiol (Risma et al., 1997). As LH per se does not promote granulosa cell proliferation (Robker and Richards, 1998a,b), the large number of granulosa cells in the prematurely developed large follicles of mice overexpressing
LH (Risma et al., 1997) or lacking ERα (present report) is likely to reflect estradiol-induced mitogenic effects (reviewed in Robker and Richards, 1998b). In contrast, ERαβKO prepubertal antral follicles are markedly deficient in granulosa cells. Therefore, the proliferative response of prepubertal granulosa cells to estrogens could be mediated by ERβ.

Following administration of exogenous gonadotropins, approximately half of our prepubertal ERβKO mice fail to form preovulatory follicles, supporting the conclusion that ERβ indeed plays an essential role in mediating the stimulatory effects of estrogens on granulosa cell proliferation. However, half of our prepubertal ERβKO mice ovulate following administration of gonadotropins. As all ‘superovulated’ ERα+/−/ERβKO mice are unable to form preovulatory follicles, functional compensation of ERβ inactivation by ERα within the ovary provides the simplest explanation to account for the existence of two groups of prepubertal ERβKO mice differing in their response to gonadotropin treatment. This functional compensation (redundancy) may be cell autonomous (Tetsuka et al., 1998) or involve paracrine interactions between granulosa and theca cells (Schomberg et al., 1999). Loss-of-function mutations of the human gene for ERβ have yet not been described. Such mutations could result in estrogen resistance limited to the ovary, and therefore in a state of female hypofertility whose expressivity within the same family might be subjected to considerable variations due to varying degrees of functional compensation by ERα.

Adult ERαKO ovaries are devoid of corpora lutea, similarly to αERKO mice (Lubahn et al., 1993; Schomberg et al., 1999). However, superovulation treatment of all prepubertal ERαKO and ERαKO/ERβ+− females elicits the formation of a large number of follicles displaying cumulus expansion, which is the last step that can be histologically identified before follicle rupture. Therefore, even though ERα is required for follicle growth in the ovaries lacking ERβ, it appears to be dispensable for this growth in WTs, whereas it is indispensable for ovulation. Whether this effect of ERα on ovulation is mediated by the theca or interstitial cells (i.e., the main sites of ERα expression in the ovary; Schomberg et al., 1999) or components of the hypothalamic-pituitary axis expressing ERα (Kuiper et al., 1997) is unknown (see Rosenfeld et al., 2000).

Interestingly, up to 83% of the αERKO mice (Lubahn et al., 1993), but only 14% of our ERαKO mice are able to superovulate (Couse et al., 1999a; Rosenfeld et al., 2000; and our present results). This discrepancy might be related to differences in genetic backgrounds. Alternatively, it could reflect the presence of a residual ERα activity in αERKO mice (Couse et al., 1995; see Results).

Interstitial glandular cells are similarly decreased in the ovarian medulla of ERαKO and ERαβKO adults. In contrast, hypertrophy of interstitial cells has been described in αERKO
mice (Schomberg et al., 1999; Couse and Korach, 1999). A possible explanation for this discrepancy could be that αERKO mice have retained some ERα activity (see above), allowing ovarian interstitial cells to respond to excess LH (Risma et al., 1997). In any event, the interstitial glandular cell deficiency in our ERαKO mice, and the normal presence of the ERα protein in these cells (Schomberg et al., 1999) strongly suggest they are primary targets of ERα action.

**Involvement of mast cells in the generation of polycystic ovaries in ERαKO and ERαβKO mutant mice**

In αERKO mice, the polycystic ovarian phenotype is correlated with an increase in serum LH (Couse et al., 1999a). Identical polycystic ovarian phenotypes are observed in ERαKO and ERαβKO mice showing that expression of ERβ in granulosa cells is not required to mediate the effects of supra-physiological levels of LH on the formation of follicular cysts, in contrast to a previous proposal (Couse et al., 1999a). Administration of either LH or histamine increases ovarian blood flow and capillary permeability, and causes edema of the ovarian interstitial tissue (Krishna et al., 1989; Jones et al., 1994 and references therein), and there is evidence relating cystic ovarian follicle formation with hypersecretion of either LH (Risma et al., 1995, 1997) or histamine (Hunter and Leathem, 1968). We show here that our ERαKO and ERαβKO mutants have numerous intraovarian mast cells, which is most probably associated with an increase in intraovarian histamine levels (Krishna et al., 1989 and references therein). It has recently been shown that the polycystic αERKO ovarian phenotype can be prevented by decreasing the serum LH levels to within the wild-type range through treatment with Antide, a gonadotropin-releasing hormone antagonist (Couse et al., 1999a). It is noteworthy, however, that (1) Antide per se can interfere with histamine secretion from mast cells (Ljungquist et al., 1988) and (2) administration of LH can increase mast-cell number in the ovary (Jones et al., 1994). It is therefore possible that locally increased histamine levels participate to the formation of follicular cysts and edema of the interstitial tissue of our ERαKO and ERαβKO ovaries. It would be interesting to know the status of intraovarian mast cells in ArKO mice, which, similar to ERαβKO mice, display both a block in estrogen signaling and high serum LH levels, but no polycystic ovarian phenotype (Fisher et al., 1998).

**Transformation of granulosa cells into Sertoli cells in the absence of estrogen receptors**

Seminiferous tubules can develop from fetal ovaries that are either (1) transplanted under the kidney capsule, (2) cultured in vitro, (3) exposed to supraphysiological concentrations of AMH in vivo or (4) lack expression of Wnt4 (Taketo-Hosotani et al., 1985; Behringer et al., 1990; Vigier et al., 1987; Vainio et al., 1999). However, the ERαβKO condition is unique in that it represents the only example of Sertoli cell differentiation occurring in a sexually mature ovary. It is noteworthy that the absence of estrogen signaling is apparently not sufficient to account for the formation of ovarian Sertoli cells, as such cells are not observed in ArKO mice that lack the capacity to synthesize estradiol (Fisher et al., 1998).

Abnormal cells present in the ovaries of αβERKO mice were characterized as Sertoli cell on the basis of their arrangement into tubular structures, their expression of AMH and SGP2, and increased Sox9 transcripts in the whole ovary (Couse et al., 1999b). However, these features are not sufficient on their own to unambiguously identify Sertoli cells as many tumors of the mouse ovary (including adenomas, as well as granulosa cell and Sertoli cell tumors) have tubular patterns (Morgan and Alison, 1987). Moreover, AMH and SGP2 are both expressed in Sertoli and granulosa cells (Ahuja et al., 1994; Aronow et al., 1993; Münsterberg and Lovell-Badge, 1991; Hirobe et al., 1992 and references therein), and Sox9 transcripts are present in WT ovary (Couse et al., 1999b). In contrast, ectoplastic specializations evidenced in ERαβKO ovaries (present report) correspond to intercellular cell junctions that, in the male, form the blood-testis barrier and are specific to Sertoli cells (Pelletier and Byers, 1992).

In αβERKO ovaries (Couse et al., 1999b), Sertoli cells are all organized into spherical structures resembling follicles and they begin to differentiate within preantral follicles. In contrast, in our ERαβKO mice, the majority of the ovarian Sertoli cells form irregular clusters (Fig. 3g,k, and data not shown), and these cells develop within the granulosa cell layers of follicles at advanced stage of atresia, most of which have already lost their spherical shape (as well as their oocyte) and exhibit leakage of their epithelial cells in the interstitial tissue. One possibility would be that Sertoli cells escaping from atretic follicles give rise to irregular aggregates, whereas those remaining within the limits of the follicular basement membrane form tubular structures. In any event, in our ERαβKO mice, Sertoli cell differentiation is clearly correlated with follicular atresia. Moreover, the observation that oocyte loss can lead to sex reversal of follicular cells (Vainio et al., 1999 and references therein) suggests that granulosa cells lacking ERs may be biased towards a Sertoli cell fate that becomes obvious only following the death of the oocyte.

In ERαβKO ovaries, all cells synthesizing large amounts of AMH possess morphological features of granulosa cells, whereas expression of AMH in ovarian Sertoli cells is low or absent. These data strongly suggest that ERαβKO ovarian Sertoli cells are derived, by metaplastic transformation, from AMH-expressing granulosa cells rather than from a bipotent precursor present within the follicular epithelium. Additionally, immunohistochemical analysis of ERαβKO ovaries shows that the number of stained follicles is reduced: the increase in AMH mRNA levels in αβERKO ovaries reported by Couse et al. (1999b) is apparently not reflected at the protein level. It seems therefore unlikely that an elevation of AMH levels is the cause of the Sertoli cell differentiation occurring in ovaries lacking ERs.

**A function for ERβ in female genital tract**

As already mentioned, the incomplete penetrance of impaired folliculogenesis in ERβKO ovaries probably reflects the existence of a partial functional compensation by ERα, which may be expressed at low levels in granulosa cells (Tetsuka et al., 1998). Inversely, our data indicate that, although ERα is the predominant ER expressed in the uterus and vagina, ERβ could partially compensate for the loss of ERα in the genital tract, as the ERαKO uterine and vaginal hypoplasia is exacerbated in ERαβKO mice, whereas the ERβKO genital tract appears normal. This possibility is in keeping with the
detection of low levels of ERβ transcripts and proteins in the uterus and vagina in both WT and αERKO mice (Couse et al., 1997; Wang et al., 1999). The lack of effect of ERβ inactivation on the genital tract of αERKO mice (Couse et al., 1999b) supports the possibility of a persistent ERα activity in αERKO mice (Couse et al., 1995).

**Inactivation of ERβ does not elicit hyperplasia of the urinary bladder and prostate gland epithelia**

Estrogens have been implicated in the pathogenesis of benign prostatic hyperplasia (reviewed in Habenicht et al., 1993), as well as in prostatic carcinogenesis and tumor progression (Bonkoff et al., 1999 and references therein). ERα and ERβ mRNA are present in approximately equal amounts in the mouse prostate (Couse et al., 1997). In addition, the ERβ protein is present at apparently high levels in the epithelium of the mouse urinary bladder (Rosenfeld et al., 1998). In this context, it is noteworthy that our data do not confirm the previously reported epithelial hyperplasia of the urinary bladder and prostate ducts in βERKO mutants (Krege et al., 1998). Moreover, in old ERαβKO males, we did not observe any significant increased cell proliferation in these epithelia.

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