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Generation of C*yp17*iCre transgenic mice and their application to conditionally delete estrogen receptor alpha (*Esr1*) from the ovary and testis

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Abstract

A transgenic mouse line that expresses iCre under regulation of the Cytochrome P_{450} 17α-hydroxylase/17, 20-lyase (*Cyp17*) promoter was developed as a novel transgenic mouse model for the conditional deletion of genes specifically in the theca/interstitial cells of the ovary and Leydig cells of the testis. In this report we describe the development of *Cyp17*iCre mice and the application of these mice for conditional deletion of the estrogen receptor alpha (*Esr1)* gene in the theca/interstitial and Leydig cells of the female and male gonad, respectively. These mice will prove a powerful tool to inactivate genes in the gonad in a cell-specific manner.

Targeted gene deletion has become a powerful tool in the study of gene function with the utilization of this technology leading to marked progress in our understanding of both physiological and pathophysiological systems. The ovary is one organ where targeted genetic deletion has proven fruitful with the establishment of transgenic mice with Cre expression targeted to granulosa cells (Lécureuil *et al.*, 2002), somatic cells (Bingham *et al.*, 2006) and the oocyte (Lan *et al.*, 2004; Lewandoski *et al.*, 1997). However, our understanding of ovarian function cannot advance at optimal pace without a tool to specifically delete genes of interest from the other major endocrine cell population of the ovary, the theca/interstitial cells. Hence, our primary goal was to develop the mice necessary to allow the specific deletion of genes in the theca/interstitial cells of the ovary and in this report we describe the generation of such a line of transgenic mice, with codon-improved Cre (iCre) driven by the promoter to Cytochrome P_{450} 17α-hydroxylase/17, 20-lyase (*Cyp17*).

Cytochrome P_{450} 17α-hydroxylase/17, 20-lyase, the product of *Cyp17* gene expression, plays a major role in the control of sex steroid hormone synthesis by mediating the17α-hydroxylation of pregnenolone or progesterone to dehydroepiandrostenedione or androstenedione, respectively. In the female mouse, *Cyp17* expression is primarily restricted to the ovary and placenta (Su *et al.*, 2002) and within the ovary, *Cyp17* is abundant in the gonadotropin-primed theca/interstitial cell population, but not the granulosa cells or oocyte (Zhang *et al.*, 2001), making it ideal for iCre targeting. Furthermore, coincident to the need of a transgenic mouse

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line with iCre targeted to the theca/interstitial cells, is one also designed to allow the deletion of Leydig cell specific genes from the male gonad. Fortunately, the specificity of *Cyp17* expression to the Leydig cells of the testis (Zhang *et al.*, 2001) makes this line of mice also ideal as a tool to inactive genes specifically within that endocrine population of cells. Hence, this report was widened as a functional characterization of these mice inclusive to both the sexes.

Three lines of *Cyp17*iCre founder mice were generated (A, B and C) by pronuclear injection of a KpnI/SalI DNA fragment derived from a *Cyp17*iCre expression plasmid (Figure 1). Then, to facilitate expression analysis, founder mice were crossed with the iCre reporter strain (Gt [ROSA]26Sor^{tm1Sor}; ROSA26) (Soriano, 1999). These mice have the *lacZ* reporter gene expressed when iCre excises a loxP-flanked polyadenylation sequence. Therefore, with iCre/ loxP recombination, only tissues with functional iCre activity will express β-galactosidase which stains intensely blue to X-gal (Bell *et al.*, 2005). Analysis of X-gal staining in the ovaries and testis collected from each of the three lines of *Cyp17*iCre/ROSA26 mice revealed no dramatic differences in the pattern of staining, however extra-gonadal X-gal staining was observed in two of the three lines. In line B, mottled X-gal staining was observed in the lungs of adult mice and in line C, disperse X-gal staining was noted in embryos collected 16.5 days after conception. These two lines are described in only a limited fashion hereafter and were not evaluated for the functional analysis (generation of *Esr1* $^{flox/flox}$*Cyp17*iCre transgenic mice), described below.

In the *Cyp17*iCre (line A) female, gross analysis of the ovary, oviduct and uterus after staining of these tissues as an intact unit with X-gal revealed strong ovarian, weak oviductal and very sparce uterine expression of β-galactosidase (Figure 2). Further histological evaluation of gonadotropin-primed *Cyp17*iCre/ROSA26 ovaries revealed X-gal staining throughout the majority of the theca/interstitium with minor staining observed in some granulosa cells in the ovary (Figure 2). In the *Cyp17*iCre/ROSA26 testis, X-gal staining was observed specifically in the Leydig cells (Figure 2). Evaluation of fetal gonadal X-gal staining on embryonic day 16.5 revealed strong staining in the testis but not the ovary (Figure 2), consistent with the pattern of expression of mRNA for *Cyp17* in these tissues (Heikkila *et al.*, 2002).

Gross morphological analysis of the placenta and embryos collected from pregnant females on embryonic day 16.5 revealed the expected placental staining (Arensburg *et al.*, 1999; Durkee *et al.*, 1992) (Figure 3). Similar to the sexually dimorphic staining of X-gal to the embryonic gonads, distinct staining was observed in the male but not the female embryonic adrenal (Figure 3). However, some inconsistency is apparent in the reported expression levels of mRNA for *Cyp17* in the male versus female embryonic adrenal (Heikkila *et al.*, 2002; Keeney *et al.*, 1995). Within the pituitary gland, X-gal staining was apparent in the intermediate lobe whereas the anterior and posterior lobes remained free of expression (Figure 3), an unexpected finding that was consistent among the three original founder lines. Gross morphological analysis of the kidney, adrenal gland, spleen and whole brain revealed only occasional and very sparse staining. Minor mottled staining was observed in some areas of the lung, whereas the liver stained intensely blue (a consistent finding among the three transgenic lines). Whether the extra-gonadal expression of iCre to the liver translates to aberrant metabolic function is unlikely though as all transgenic mice maintained good health and developed at the expected rate of growth. Overall, X-gal staining indicated relatively specific incorporation of iCre.

To determine the functional ability of iCre to excise a floxed gene *in vivo*, *Cyp17*iCre mice was crossed with those having loxP sites flanking estrogen receptor alpha (*Esr1*) (Gieske *et al.*, 2008), with the goal of specifically deleting *Esr1* from the ovarian theca/interstitium and testicular Leydig cells. Because *Esr1* is localized to the theca/interstitial cells (Schomberg *et al.*, 1999) and the surface epithelium of the ovary (Urzua *et al.*, 2006), these mice were well

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suited to test the ability to delete a gene specifically from our targeted population of cells. Furthermore, total genetic deletion of *Esr1* leads to the development of severe hemorrhagic ovarian cysts (Dupont *et al.*, 2000), making mice with a conditional deletion of this gene an excellent model for further analysis of the role of *Esr1* in ovarian health and reproductive function. Founder lines had also undergone multiple generations of breeding before crossing with *Esr1* $^{flox/flox}$ mice, allowing analysis of functional excision via a transgene with a now stable insertion. Briefly, *Esr1*$^{flox/flox}$ mice were created by a targeting strategy used to generate *Esr1*$^{-/-}$ mice (Dupont *et al.*, 2000), as described previously (Gieske *et al.*, 2008). To generate *Esr1*$^{flox/flox}$ *Cyp17*iCre mice, *Esr1*$^{flox/flox}$ mice were first crossed with *Cyp17*iCre mice. *Esr1*$^{flox/+}$*Cyp17*iCre F1 heterozygotes were then bred with *Esr1*$^{flox/flox}$ mice to generate four potential genotypes: *Esr1*$^{flox/flox}$ *Cyp17*iCre (the desired transgenic), as well as *Esr1*$^{flox/+}$*Cyp17*iCre, *Esr1*$^{flox/flox}$ and *Esr1*$^{flox/+}$. Analysis of *Esr1* $^{flox/flox}$ *Cyp17*iCre mice indicated successful application of the *Cyp17*iCre line as a conditional deleter of *Esr1* within the gonad. Both in the ovary and testis of *Esr1* $^{flox/flox}$ *Cyp17*iCre mice, deletion of *Esr1* was restricted to the gonads and abundant Esr1 expression was maintained in the uterus, oviduct, and epididymis, respectively (Figure 4).

Within the ovary, normal Cyp17 protein expression was observed by immunohistochemical analysis in the theca/interstitial cells of both *Esr1* $^{flox/flox}$ and *Esr1* $^{flox/flox}$*Cyp17*iCre mice (Figure 5). In contrast, Esr1 expression, while apparent in the theca/interstitial cells and ovarian surface epithelium of *Esr1* $^{flox/flox}$ mice, was only detected in the ovarian surface epithelium of *Esr1* $^{flox/flox}$*Cyp17*iCre mice (Figure 5), indicating successful deletion of *Esr1* from the iCre-expressing theca/interstitium and maintained expression in the non-targeted ovarian surface epithelium. Similar results were observed in the testis. Normal Cyp17 protein expression was observed in the Leydig cells of both *Esr1* $^{flox/flox}$ and *Esr1* $^{flox/flox}$*Cyp17*iCre mice (Figure 5). Esr1 expression was detected in the Leydig cells of *Esr1* $^{flox/flox}$ mice but not in *Esr1* $^{flox/flox}$*Cyp17*iCre transgenic mice (Figure 5), again indicating successful deletion of *Esr1* from iCre-expressing cells after excision of the loxP sequence.

In summary, we report the development and application of a novel strain of transgenic mice with iCre targeted to the *Cyp17* promoter. The use of these mice to conditionally delete genes of interest from the theca/interstitial cells of the ovary and Leydig cells of the testis will allow rapid advancement of our understanding of gene function as it relates to fertility and reproductive health.

METHODS

Generation of Cyp17iCre Transgenic Mice

Three transgenic mouse lines were constructed that expressed iCre recombinase under the control of the mouse *Cyp17* promoter (*Cyp17*iCre). An iCre fragment was amplified from the iCre plasmid (pBlue.iCre, generously provided by Dr. R. Sprengel, Max-Planck Institute for Medical Research, Germany) by polymerase chain reaction (PCR), digested and subcloned into another plasmid (pGL3B-iCre) to generate a promoter-free iCre expression cassette (Shimshek *et al.*, 2002). Genomic DNA fragments corresponding to the promoter region of *Cyp17* were amplified from mouse (C57-B6) genomic DNA, and cloned into the promoter-free iCre expression cassette (pGL3B-iCre) to produce a *Cyp17*iCre expression plasmid (pGL3B-*Cyp17*iCre). The nucleotide sequences of this transgene vector, pGL3B-*Cyp17*iCre, were confirmed by sequence analysis. A KpnI/SalI DNA fragment from pGL3B-*Cyp17*iCre vector which contained the 3.34 kb *Cyp17* promoter, a 1.1 kb iCre coding sequence and a SV40 late poly A signal (Schorpp et al., 1996) was cut, purified and microinjected into the pronuclei of fertilized eggs from C57-B6/SJL mice as previously described (Jorgez *et al.*, 2006; Lan *et al.*, 2004; Li *et al.*, 2005; Shimshek *et al.*, 2002). The microinjected eggs were transferred to

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pseudopregnant mothers, yielding three viable *Cyp17*iCre founder mice that transferred this transgene to their progeny.

Animal use and Genotyping

Genomic DNA was extracted from ear punches or embryonic tails using the Easy DNA kit (Invitrogen), according to the manufacturer's directions. The presence of iCre or LacZ was determined by PCR using the following primer pairs: Cre-F (5′-tct gat gaa gtc agg aag aac c-3′) and Cre-R (5′-gag atg tcc ttc act ctg att c-3′); LacZ-F (5′-gcg tta ccc aac tta atc g-3′) and LacZ-R (5′-tgt gag cga gta aca acc-3′) (Jorgez *et al.*, 2006). For the functional analysis, the primer sets ERαP2F (5′-gtg tca gaa aga gac aat-3′) and ERαP3 (5′-ggc att acc act tct cct ggg agt ct-3′) were used to determine the presence or absence of loxP sequences and the primer sets ERαP1 (5′-ttg ccc gat aac aat aac at-3′) and ERαP3 were used to determine whether or not exon 3 had been deleted (Gieske *et al.*, 2008). Animal use was approved by the respective University of Kentucky and Baylor College of Medicine Animal Care and Use Committees.

Histochemical Analysis

X-gal staining for β-galactosidase expression was performed on fresh tissues collected at sacrifice with the reagents and protocol recommended for direct staining by the manufacturer (Speciality Media, NJ). Briefly, tissues were fixed with the supplied paraformaldehyde based-fixative for 1 h on ice, rinsed with two PBS-based rinse solutions at room temperature and then incubated overnight at 37°C in the dark with X-gal stock solution diluted 1:40 in the supplied base solution. After the overnight incubation, tissues were washed and embedded in either paraffin or OCT. Paraffin-embedded sections were cut to 7 μm, deparaffinized by treatment with xylenes, rehydrated through a graded series of alcohols then rinsed and counterstained with nuclear fast red. Tissues embedded in OCT were cut to 10 μm, rehydrated and counterstained with nuclear fast red.

Immunohistochemical analysis of Esr1 and Cyp17 was performed on ovaries and testis collected at sacrifice, fixed with 4% neutral buffered paraformaldehyde and then embedded in paraffin blocks. Sections were cut to 4 μm and immunostaining performed on deparaffinized sections using an EnVision detection kit (DAKO, Carpenteria, CA), according to the manufacturer's instructions. Antigen-retrieval was perfomed using 10 mM citrate buffer (pH 6.0) in an autoclave for 15 min at 120°C and the endogenous peroxidase activity was blocked with 5% hydrogen peroxide for 15 min. Sections were then incubated with primary antibodies that recognized Esr1 (mouse monoclonal 6F11, 1:40 dilution, Novocastra, Newcastle, UK), and Cyp17 (rabbit polyclonal, 1:100 dilution, kindly provided by Dr. A.J. Conley, Department of Population Health and Reproduction, University of California-Davis (Pattison *et al.*, 2007)). 3,3′-diamino-benzidine (DAB, DAKO) or 3-amino-9-ethylcarbazole (AEC, DAKO) were then used as a chromogen and counterstaining was accomplished with Mayer's hematoxyline. Staining was examined using a BX51 microscope (Olympus, Japan) with images acquired using the DP-70 imaging system (Olympus).

ACKNOWLEDGMENTS

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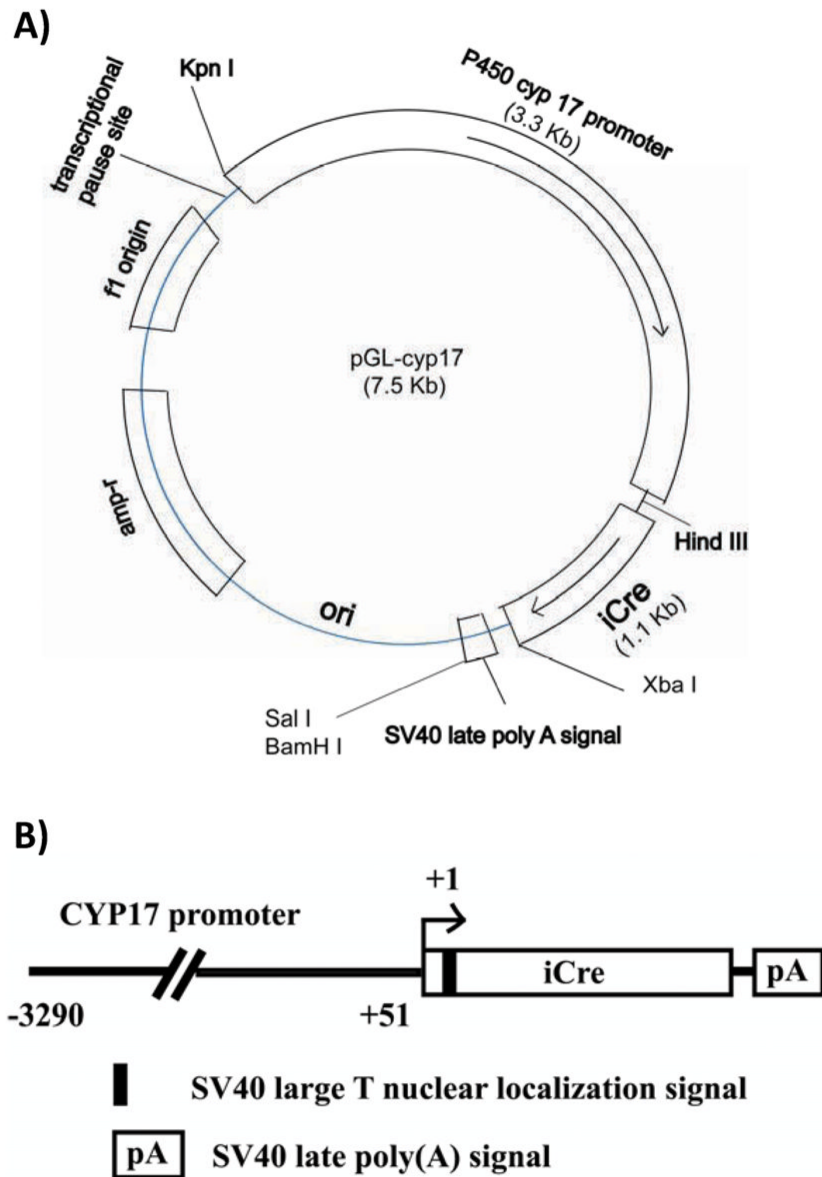

Figure 1.
Schematic representation of the *Cyp17*iCre transgene construct. A) Plasmid map of the completed *Cyp17*iCre construct. B) Linearlized structure of Kpn 1 /Sal 1 fragment of the construct. This DNA fragment was used for oocyte injection.

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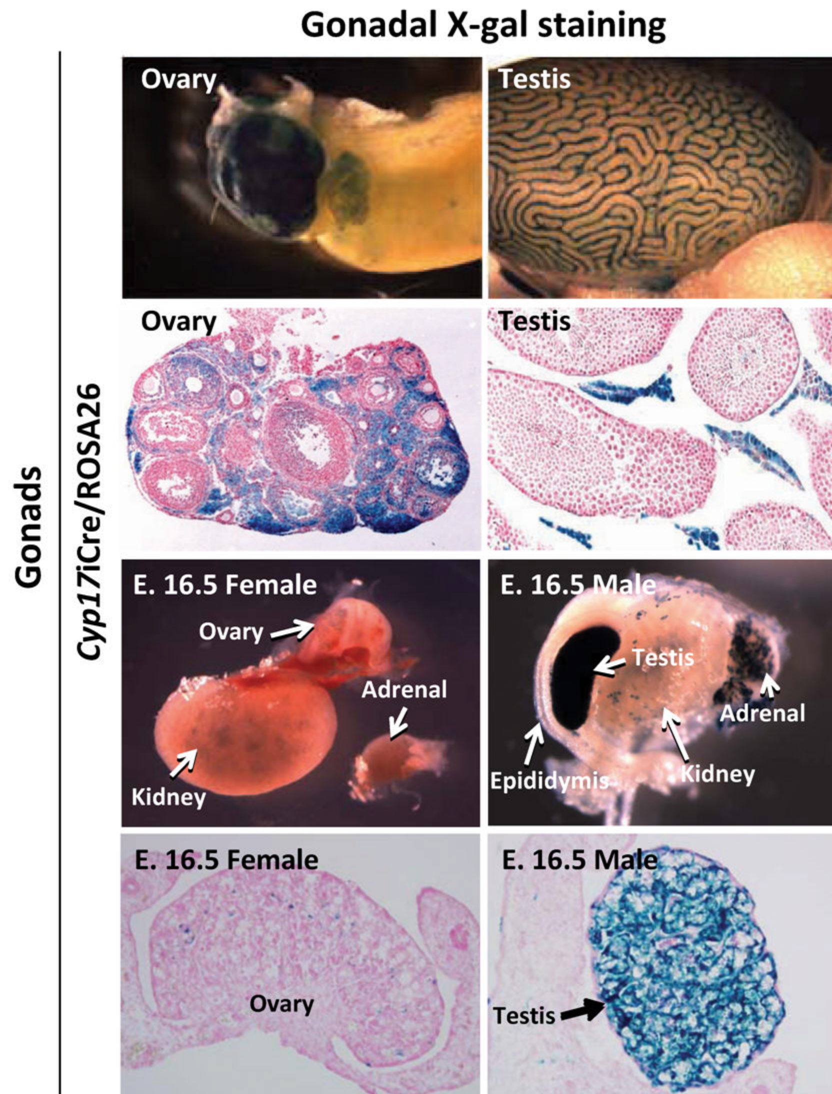

Figure 2.

β-galactosidase expression in gonadal tissues of *Cyp17*iCre/ROSA26 mice. Upper panels: Direct X-gal staining of the adult female and male gonads. Sectioned images of the ovary and testis are shown. The sections were counterstaining with nuclear fast red. Lower panels: Direct X-gal staining of embryonic day 16.5 female and male gonads. Sectioned images of the ovary and testis are shown. The sections were counterstaining with nuclear fast red.

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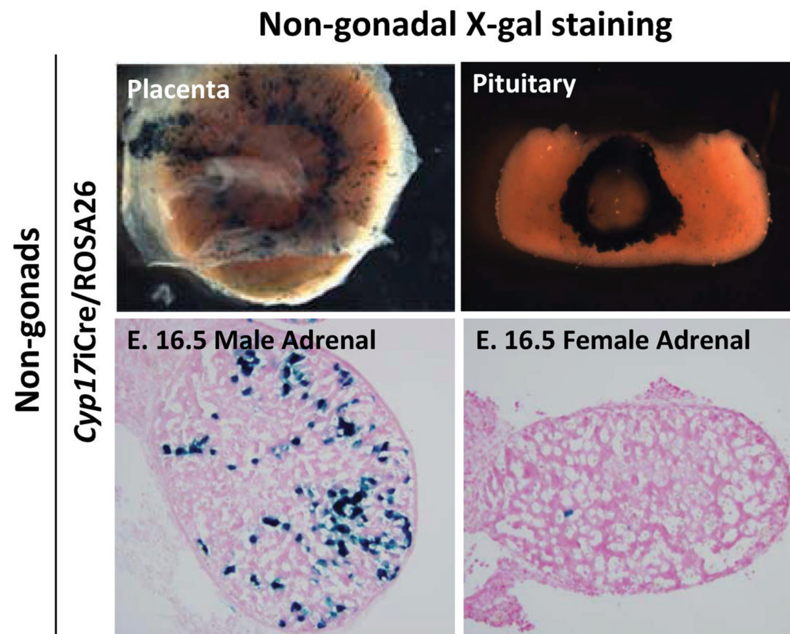

Figure 3.
β-galactosidase expression in non-gonadal tissues of *Cyp17*iCre/ROSA26 mice. Upper panels: Direct X-gal staining of the pituitary and placenta of a female mouse. Lower panels: Direct X-gal staining of the embryonic day 16.5 adrenal gland. Sectioned images of the male and female embryonic adrenal gland are shown. The sections were counterstaining with nuclear fast red.

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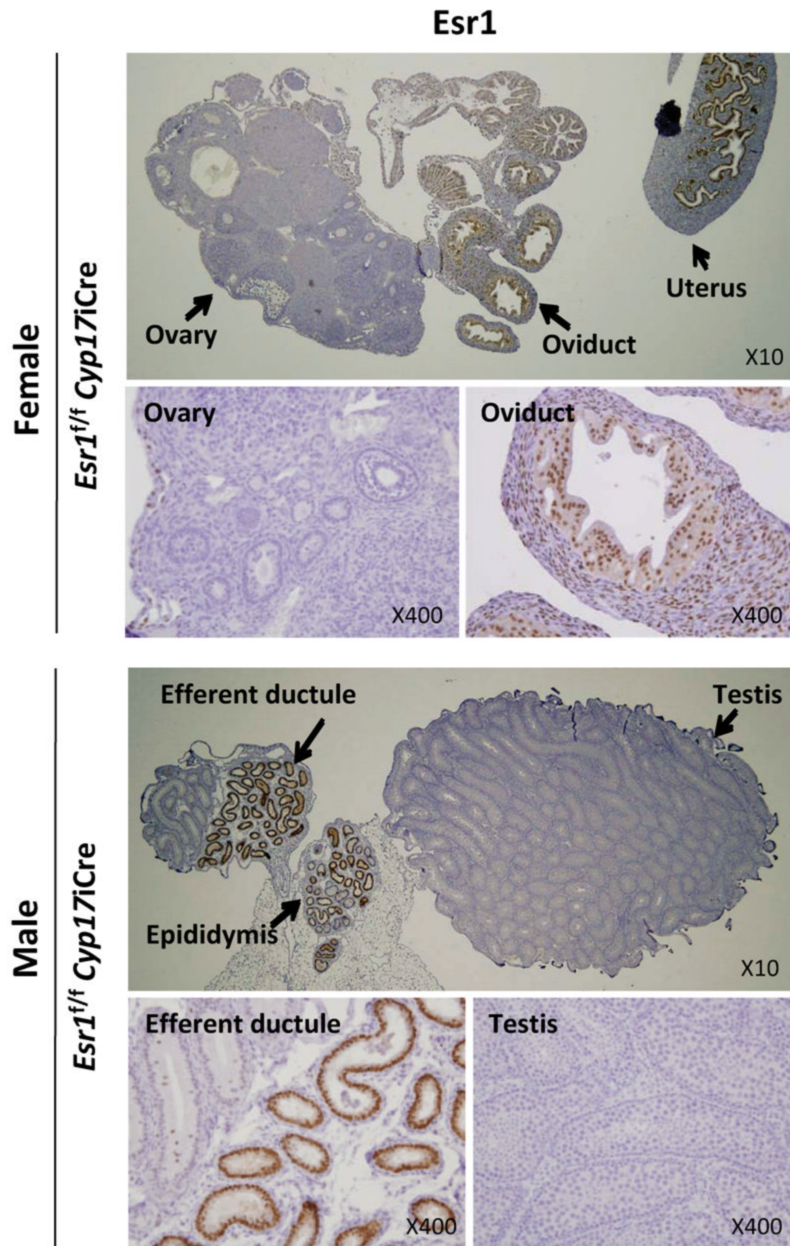

Figure 4.

Gonad-specific deletion of *Esr1* gene by *Cyp17*iCre mice. Upper panels: Expression of Esr1 in the reproductive tissues of a female *Esr1* $^{flox/flox}$ *Cyp17*iCre mouse. Note strong Esr1 expression both in oviduct and uterus but not in the ovary except for ovarian surface epithelial cells. Lower panels: Expression of Esr1 in the reproductive tissues of a male *Esr1* $^{flox/flox}$ *Cyp17*iCre mouse. Note strong Esr1 expression in the efferent ductile and epididymis but not the testis.

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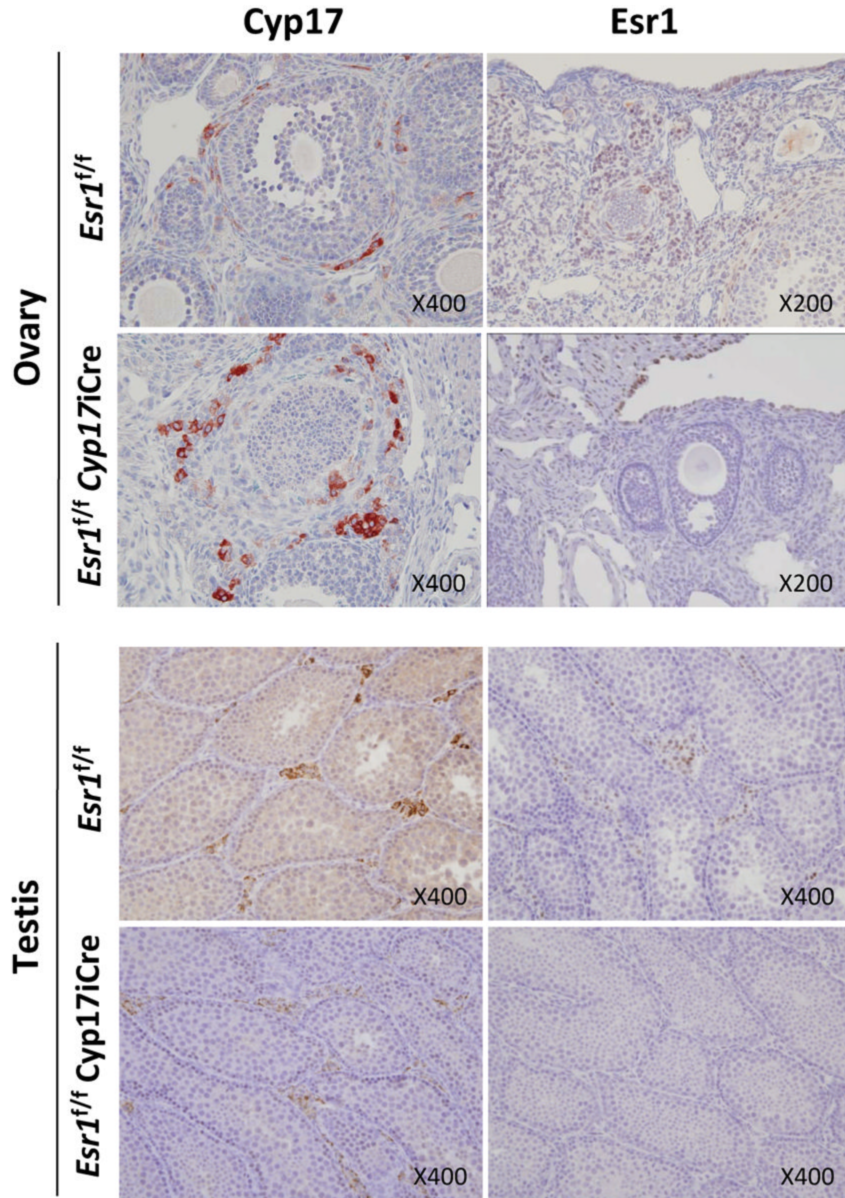

Figure 5.

Successful deletion of Esr1 by the application of *Cyp17*iCre mice. Upper panels: Expression of Cyp17 and Esr1 in the ovary of *Esr1* $^{flox/flox}$ and *Esr1*$^{flox/flox}$ *Cyp17*iCre mice. Note that Esr1 expression is absent in the theca/interstitial cells of the *Esr1*$^{flox/flox}$ *Cyp17*iCre mouse ovary while Cyp17 expression persists. Lower panels: Expression of Cyp17 and Esr1 in the testis of *Esr1* $^{flox/flox}$ and *Esr1*$^{flox/flox}$ *Cyp17*iCre mice. Note that Esr1 expression is absent in the Leydig cells of the *Esr1*$^{flox/flox}$ *Cyp17*iCre mouse testis while Cyp17 expression persists.