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Generation and characterization of an estrogen receptor alpha-iCre knock-in mouse

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Summary

Two estrogen receptors, ESR1 and ESR2, are responsible for the classical actions of estrogens in mammalian species. They display different spatiotemporal expression patterns and nonoverlapping functions in various tissues and physiological conditions. In this study, a novel knock-in mouse line that expresses codon-improved Cre recombinase (iCre) under regulation of the natural *Esr1* promoter (*Esr1*-iCre) was developed. Functional characterization of iCre expression by crossing them with reporter lines (ROSA26-lacZ or Ai9-RFP) showed that iCre is faithfully expressed in *Esr1*-lineage cells. This novel transgenic mouse line will be a useful animal model for lineage-tracing *Esr1*-expressing cells, selective gene ablation in the *Esr1*-lineage cells and for generating global *Esr1* knockout mice.

Keywords

BAC clone; estrogen receptor alpha; iCre recombinase; transgenic

1 | INTRODUCTION

17 β -estradiol (E2) is a steroid hormone that regulates a plethora of reproductive, metabolic, immunologic, cognitive, and skeletal functions (Arnal et al., 2010; Heldring et al., 2007; Kalaitzidis & Gilmore, 2005; Wise, Dubal, Wilson, Rau, & Bottner, 2001). Two estrogen receptors, estrogen receptor alpha (ERa or ESR1) and beta (ERb or ESR2) mediate classical actions of E2 in most of mammalian species (Heldring et al., 2007; Katzenellenbogen et al., 2000). These two receptors display nonoverlapping expression patterns in specific tissues

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CONFLICT OF INTEREST

The authors declare no conflict of interest here.

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SUPPORTING INFORMATION

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(Kuiper, Enmark, Peltö-Huikko, Nilsson, & Gustafsson, 1996; Kuiper & Gustafsson, 1997; Warner, Huang, & Gustafsson, 2017). For example, in the adult ovary, ESR1 is predominantly expressed in theca cells and surface epithelium, whereas ESR2 is exclusively expressed in granulosa cells (Sar & Welsch, 1999). In the brain kisspeptin neurons express ESR1 whereas in the hypothalamus GnRH neurons express ESR2 (Ng, Wolfe, Novaira, & Radovick, 2009; Radovick, Levine, & Wolfe, 2012). Functionally, ESR1 and ESR2 seem to mediate E2 actions that are not redundant. For instance, in the ovary, ESR2 but not ESR1 plays a crucial role for follicle growth and maturation (Emmen et al., 2005; Hegele-Hartung et al., 2004). In contrast, in the brain, ESR1 but not ESR2 provides protection against ischemic brain injury (Dubal et al., 2001, 2006; Garcia-Segura, Azcoitia, & DonCarlos, 2001; Raval, Bhatt, & Saul, 2009; Wise, Dubal, Rau, Brown, & Suzuki, 2005). However, it is not known when and how each cell selectively expresses ESR1 or ESR2. Use of transgenic animals that permit lineage tracings for ESR1-vs. ESR2-expressing cells during normal or pathogenic development may provide clues for such selective estrogen receptor expression.

In this study, a novel knock-in mouse line that expresses codon-improved Cre recombinase (iCre) under regulation of the *Esr1* promoter (*Esr1*-iCre) was generated and crossed with ROSA26-lacZ or Ai9-RFP reporter mice (Madisen et al., 2010; Soriano, 1999). Characterization of these two double transgenic lines showed that the *Esr1*-iCre mouse excises floxed sequences and induces reporter gene expression faithfully in the *Esr1*-lineage cells. In addition, characterization of homozygous *Esr1*-iCre showed that they exhibit phenotypes that are identical to those of global *Esr1* knockout mice.

2 | METHODS

2.1 | Ethics statement

This study was carried out in accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal protocols were approved by the University of Illinois Animal Care and Use Committee (Protocols: 11205 and 14222), and all efforts were made to minimize animal suffering. Animal models generated in this study will be made readily available to the research community.

2.2 | Targeting vector construction

An iCre-polyA-FRT-neo-FRT cassette (3,266 bp) was generated from a pBluescript KS(+)-iCre plasmid as described in a previous transgenic study (Cacioppo et al., 2016) and was modified for insertion into the mouse *Esr1* gene (Figure 1a). The cassette was inserted before the translational initiation codon of the *Esr1* gene of a BAC clone (ID RP23-7D5) that was purchased from Invitrogen (Carlsbad, CA) by recombineering according to protocols provided by Frederick National Laboratory for Cancer Research (Jahrling et al., 2014; Parkitna, Engblom, & Schutz, 2009; Thomason, Sawitzke, Li, Costantino, & Court, 2014). Briefly, a homology arm (upstream from ATG at exon 2) was amplified using primer pairs (5'-GCA GCG TGC TTC CAG GTT TGT CTA A-3', 5'-GAA GCT TGG TAA GTG GCA GCC GGC G-3') and cloned between the *MluI* site and the *HindIII* site upstream of the cassette. Another homology arm downstream from ATG was amplified using primer

pairs (5'-AGG CCG AGG CGG CCA TGA CCA TGA CCC TTC ACA C-3', 5'-AGA CGT CCG GCG TTG AAC TCG TA-3') and cloned between the *SfiI* site and the *AadI* site after the cassette. The cassette with the two homologies was cut with *MluI* and *AadI*, gel purified, and introduced into *E. coli* SW106 (heat-induced) carrying the BAC for insertion. Engineered BAC clones were selected and grown on LB plate media supplemented with kanamycin (25 mg/L). The engineered *Esr1* gene was retrieved in a pL253 targeting vector. An upstream homology arm (*NotI*/*HindIII* fragment) amplified with primer pairs (5'-GGC GGC CGC CTT TGG AAC AAC AAA TTT TTA TTC ATC AG-3' and 5'-GAA GCT TCC AAG AAA CAA CTC CCA AAC CCA-3') and a downstream homology arm (*HindIII*/*SpeI* fragment) amplified with the primer pairs (5'-GAA GCT TGG AGC AAA GAA GGT TGA AAG ATA C-3' and 5'-CAC TAG TCC ACT CAA ATA TGT TCT CAT AAA GAG-3') were cloned between the *NotI* site and *SpeI* site of pL253. The resulting plasmid was linearized by *HindIII* digestion and introduced into *E. coli* SW106 carrying the engineered BAC. Retrieved clones were selected and grown on LB medium supplemented with ampicillin (50 mg/L). The targeting vector was linearized by *ClaI* digestion and used for embryonic stem (ES) cell targeting (Figure 1b).

2.3 | Gene targeting

The targeting vector was introduced into the genome of mouse ES cells (C57BL/6N-PRX-B6N #1, Jackson Laboratory, Bar Harbor, Maine) at the University of Illinois at Urbana-Champaign Biotechnology Center. ES cells received the targeting vector DNA through electroporation and homologous recombination at 75% confluence and were then grown in selection media with G418 (200 mg/mL). DNA from ES cell was extracted and sent to the Murine Genetic Analysis Laboratory at the University of California, Davis. Quantitative PCR, long range PCR, and viral screening were performed on 208 ES cell clones to ensure insertion of a single copy in the correct orientation without a viral infection. Briefly, long range PCR was performed on the 5' end with primers LR-F1 and LR-vR1 (5'-ATG ACC ACG AGT GGA GGA TGA CC-3' and 5'-CCC TGA ACA TGT CCA TCA GGT TC-3') to produce 4.8 kbp DNA fragments, and on the 3' end with primers LR-vF1 and LR-R1 (5'-CAA AAA AGC CTC CTC ACT ACT TCT GG-3' and 5'-GCA TGT CTC TGC AAC AAG TTA CCT GG-3') to produce 3.6 kbp DNA fragments (Figure 1c). Four ES cell clones were identified as correctly targeted. Selection copy number analysis was performed based on the neomycin insertion sequence to ensure that no additional cassette copies were present. Cells of one of these lines were expanded and used to generate black and white chimeric mice by injecting them into blastocysts that were retrieved from C57BL/6J albino mice at the University of Illinois Biotechnology Center.

2.4 | Animals used

Chimeric mice containing the *Esr1*-iCre construct, characterized by mixed coat colors, were produced from surrogate mothers. Four male chimeras (>70% black) were bred with albino wild type (WT) C57B/6J female mice. The genotype of their offspring was determined by PCR of ear tissue using primers iCre-F (5'-TCT GAT GAA GTC AGG AAG AAC C-3') and iCre-R (5'-GAG ATG TCC TTC ACT CTG ATT C-30) (Bridges et al., 2008) to detect iCre presence. The three-primer set 5'-CTT CTC TAA TCG CAG GCT CTA CTC-3' (*Esr1*-E2F), 5'-CTT TGG TGT GAA GGG TCA TGG TC-3' (*Esr1*-E2R), and 5'-ATT

CTC CTC ATC ACC AGG GAC ACA-3' (iCreR1) was later used to determine if mice were hetero- or homozygous for *Esr1*-iCre. From the genotyping PCR, WT hetero- or homozygous for *Esr1*-iCre was clearly distinguishable in the agarose gel (Figure 1d). The albino breeder female mice gave birth to litters of expected size with numerous black-haired pups that were globally positive for the iCre gene. After the pups reached puberty, these mice were bred with B6N.Cg-Tg(ACTFLPe)9205-Dym/CjDswJ (FLP) mice (Jackson Laboratory) to remove neomycin and then back-crossed with WT mice to remove the *Flp* gene (Rodriguez et al., 2000). FLP presence was determined by PCR with the primers 5'-AAC GGA ACA GCA ATC AAG AGA GCC-3' and 5'-TGC TTC TTC CGA TGA TTC GAA CTG-3'. For characterization of expression, *Esr1*-iCre mice were bred with two reporter lines: B6;129S4-Gt26Sor^{tm1Sor}/J (ROSA26) reporter mice to visualize expression by X-gal staining (Soriano, 1999) and B6;129S6-Gt26Sor^{tm9}(CAG-tdTomato)^{Hze}/J (Ai9) reporter mice (Madisen et al., 2010) to visualize expression by the presence of red fluorescent protein (RFP).

2.5 | Characterization of *Esr1*-iCre expression

For X-gal staining, tissues, or whole embryos were incubated in 4% paraformaldehyde (PFA) on ice for 1–2 h, and then washed three times with ice-cold phosphate buffered saline (PBS). After washing, the tissues were placed in X-gal (5-bromo-4-chloro-3-indolyl-*b*-D-galactopyranoside) staining solution (Millipore Specialty Solutions, Billerica, MA) on a shaker and incubated for 24 h in the dark at 4°C. After staining, tissues/embryos were washed three times with icecold PBS and then incubated in X-gal holding solution (Millipore) for 1 h at room temperature. Stained samples were fixed again with 4% PFA at 4°C overnight. Fixed samples were washed with 70% ethanol, and images of the tissues were taken. For visualization of RFP expression, images were taken using a Zeiss Stemi SV 11 fluorescent dissecting microscope (Zeiss MicroImaging, Thornwood, NY) immediately after euthanasia.

2.6 | Comparison of the phenotype of heterozygous and homozygous *Esr1*-iCre mice

Heterozygous and homozygous *Esr1*-iCre mice were subjected to experimentation at postnatal day (PND) 38. Estrous cyclicity of mice was determined via microscopic observation of vaginal smear contents for 10 days. For histology, tissues were washed three times with ice-cold PBS and then fixed with 4% PFA at 4°C overnight. Fixed samples were washed with 70% ethanol, and images of the tissues were taken before tissues were dehydrated and embedded in paraffin. Paraffin blocks were sectioned at 6 μ m thickness using a microtome. Slides were then counter-stained with hematoxylin and imaged using an Olympus BX51 microscope.

3 | RESULTS AND DISCUSSION

This study generated a novel mouse line that has an insertion of iCre sequence in the place of the initiation codon (ATG) of the *Esr1* gene. Functional expression of iCre in the *Esr1*-iCre mice was first examined by crossing them with a reporter line (Ai9) that expresses RFP upon Cre-mediated excision of a stop sequence (Madisen et al., 2010) and localizing the RFP expression in the organs of the resulting double transgenic mice (*Esr1*-RFP) at tissue

levels. At 2 months of age, the skin of *Esr1*-RFP mice expressed RFP, making them look globally red both in males and females (Figure 2a–f).

In the male reproductive tract, where ESR1 is highly expressed (Hess, Bunick, & Bahr, 2001), strong RFP expression was seen in the epididymis, preputial gland, and prostate (Figure 2g–h). In the testis, RFP expression was observed in the interstitium where Leydig cells are located (Figure 2h; Supporting Information Figure 1A), and this was expected because of a well-established expression of ESR1 in the Leydig cells (Zhou et al., 2002). The female reproductive organs—the ovary, oviduct, and uterus where ESR1 is highly expressed—showed strong RFP expression (Figure 2i; Supporting Information Figure 1B). ESR1 is known to be expressed in theca and surface epithelial cells in the ovary (Curtis Hewitt, Couse, & Korach, 2000; Sar & Welsch, 1999) and epithelial, stromal, and smooth muscle cells throughout the uterus and oviduct including the infundibulum, ampulla, and isthmus (Shao et al., 2007; Winuthayanon, Hewitt, Orvis, Behringer, & Korach, 2010). Overall, RFP expression resembles the pattern of the reported ESR1 expression in the *Esr1*-RFP mouse.

In both the male and female brain, RFP expression was found throughout the cerebrum, cerebellum, and hypothalamus. RFP expression was also observed in the optic nerves and medulla oblongata (Figure 2j–k). In the pituitary, prominent RFP expression was visible in the anterior pituitary lobe, but only faint RFP expression was observed in the posterior pituitary (Figure 2l). In the thoracic cavity, the lungs showed weak RFP expression (Figure 2m). In the digestive tract, widely spread and punctate RFP expression was detected from the small intestine (Figure 2n). Interestingly, the RFP expression was observed in the small intestine as early as PND 3 (Supporting Information Figure 2). We then crossed *Esr1*-iCre mice with a ROSA26-lacZ line of mice to visualize *Esr1*-lineage cells at an embryonic stage by X-gal staining. The resulting mice (*Esr1*-lacZ) displayed b-galactosidase expression in the small intestine as early as embryonic day (ED) 12.5 (Supporting Information Figure 2). In the adrenal–kidney complex, strong and moderate RFP expression were detected from the adrenal and kidney, respectively (Figure 2o). RFP expression was not detected in the esophagus, spleen, adipose tissue, and circulating blood. In Table 1, the expression pattern of RFP expression in major organs of the *Esr1*-RFP mice is summarized.

Examination at ED 12.5 showed that RFP expression was detected in the mammary buds located in the pelvic skin and the gonadal region (Figure 3a). This expression is in line with the established role of ESR1 in the mammary gland elongation, branching, and ductal epithelial cell proliferation (Feng, Manka, Wagner, & Khan, 2007). RFP expression in the thorax was also detected from the cardiac atrium (but not the ventricle), lung, and thymus at ED 12.5 (Figure 3b). In the developing mouse heart, the atrium expresses ESR1 but not ESR2 from ED 9.5 to 12.5 (Lemmen et al., 1999). Therefore, lacZ expression in the atrium in mouse embryos is consistent with this previously reported ESR1 expression pattern. At ED 16.5, lacZ expression was detected from the pelvis, limbs, and digital skin. By PND 5, RFP expression was seen in the entire skin of the body (Figure 4). Expression of estrogen receptors and their functional roles are well documented. For instance, radioisotope-labeled estradiol binds to the skin of the mouse (Uzuka, Nakajima, & Mori, 1978), and ESR1 regulates the biosynthesis of hyaluronic acid and collagen biosynthesis, including collagen

production, stabilization, and degradation in the mouse skin (Markiewicz et al., 2013; Uzuka, Nakajima, Ohta, & Mori, 1980). Interestingly, in *Esr1*-iCre mouse skin, RFP expression was first seen only in the mammary bud at ED 12.5 but in the entire skin of the body by the time of birth. It will be interesting to see whether the first synthesis of hyaluronic acid and collagen coincides with the first ESR1 expression in the skin as well as which specific cell type expresses ESR1 in the skin.

In the reproductive tract, the earliest possible ESR1 expression is at ED 12.5 because X-gal staining shows a clear functional expression of iCre at this stage in the developing gonads and mesonephros (Figure 5). This expression pattern indicates that *Esr1*-lineage cells emerge before ED 12.5 in the developing gonad. As ESR1 plays many important regulatory roles in the gonads and reproductive tract tissues, it will be interesting to see when and how the *Esr1*-lineage development occurs in the reproductive organs using the *Esr1*-iCre mice.

In the adult mouse brain, ESR1 is predominantly expressed in the hippocampus, preoptic area, and hypothalamus, but it was sparse or absent from the cerebral cortex and cerebellum. Where ESR1 expression was rare, *Esr2* was abundantly expressed in the cerebral cortex and cerebellum (Mitra et al., 2003). RFP expression was not detected from the olfactory bulb and telencephalon region at ED 19.5. However, in contrast to the embryonic brain, strong RFP expression was detected at PND 20 from the cerebrum, which originates from the telencephalon. Notably, strong RFP expression was detected from the cerebral cortex region in a sagittal section of the cerebral hemisphere. Therefore, *Esr1* expression in the cerebrum occurs after birth but before PND 20. Sagittal sections of the whole-brain, pons, and medulla showed weak RFP expression. In the isocortex and prefrontal cortex of the mouse brain, *Esr1* mRNA expression gradually decreases by methylation of CpG sites of the *Esr1* promoter region during early postnatal development, whereas *Esr2* mRNA expression gradually increases (Prewitt & Wilson, 2007; Westberry & Wilson, 2012; Westberry, Trout, & Wilson, 2010). In the adult hypothalamus, *Esr1* is expressed in the arcuate nucleus (ARC) and the ventromedial hypothalamus (VMH) (Merchenthaler, Lane, Numan, & Dellovade, 2004). ESR1 has roles in metabolic regulation, including energy expenditure, leptin signaling, and food intake in the hypothalamus (Bennett et al., 1999; Diano, Kalra, & Horvath, 1998; Frank, Brown, & Clegg, 2014; Musatov et al., 2007; Xu et al., 2011). In the feedback of estradiol on hypothalamus, kisspeptin neurons express ESR1 and receive the estradiol signal directly and regulate the secretion of gonadotrophic-releasing hormone (GnRH) from the GnRH neurons (d'Anglemont de Tassigny & Colledge, 2010; Wintermantel et al., 2006). RFP expression was observed in the basal region of the hypothalamus corresponding to the ARC and VMH region at ED19.5 (Figure 6). In the fetal brain, the medial preoptic area (MPA), anterior cortical amygdaloid nucleus (ACo), and posterolateral cortical amygdaloid nucleus (PLCo) also show RFP expression in microscopic observation. In the adult mouse, expression of ESR1 in the MPA, ACo, and PLCo are well documented (Merchenthaler et al., 2004; Mitra et al., 2003). These results suggest that *Esr1* gene expression in the ARC and VMH region of the hypothalamus, preoptic area, and ACo and PLCo of the amygdala begins during the embryonic stage prior to ED 19.5. In the pituitary, only the anterior and intermediate lobes of the pituitary expressed RFP but not the posterior pituitary. Together with hypothalamic ESR1, ESR1 in the anterior pituitary has critical roles in estrogen feedback in hypothalamic-pituitary-gonadal axis. Anterior

pituitary-specific *Esr1* knockout mice revealed subfertility or infertility and lack of estrogen has a negative feedback effect on the gonadotroph (Gieske et al., 2008; Singh et al., 2009). Therefore, as described above, RFP expression in the hypothalamus and pituitary of the *Esr1*-RFP mice reflects the expression pattern of ESR1 reported in the previous studies.

Finally, the reproductive phenotype of heterozygous and homozygous *Esr1*-iCre mice was compared (Figure 7a). Because the iCre sequence was inserted in the translation initiation codon of the *Esr1* gene, homozygous knock-in mice were expected to be phenotypically the same as the global *Esr1*-knockout. In fact, homozygous female *Esr1*-iCre/iCre mice exhibited no sign of estrous cyclicity but displayed constitutive diestrus. Also, hemorrhagic follicles and cystic follicles were found in *Esr1*-iCre/iCre ovaries. In immunohistochemical localization of ESR1 proteins, no ESR1 expression was seen in the uterus of homozygous *Esr1*-iCre, whereas uterine epithelial cells and stroma cells of heterozygous *Esr1*-iCre showed ESR1 expression (Figure 7b). These reproductive abnormalities and absence of ESR1 expression in *Esr1*-iCre/iCre mice are indistinguishable from those of global *Esr1*-knockout mice (Dupont et al., 2000). Therefore, *Esr1*-iCre/iCre can be used as an alternative animal model for global *Esr1* knockouts, while retaining iCre expression in *Esr1*-lineage cells.

As RFP expression was observed in various tissues in the adults but only in a few specific cell types at younger ages, the *Esr1*-iCre mice may not be used as a deleter line or a cell-lineage tracer in adulthood but at embryonic or postnatal stages of development. Of note, this novel *Esr1*-iCre mouse line may be used along with the *Esr2*-iCre mouse that we previously generated (Cacioppo et al., 2016) for a lineage-tracing ESR1-vs. ESR2-expressing cells and for a selective deletion of genes in respective cell lineages.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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FIGURE 1.

Construct of targeting vector, screening of targeted ES cells and genotyping of *Esr1*-iCre mice. (a) Strategy for gene targeting. A cassette containing the coding region of iCre, SV40 late polyadenylation signal, and FRT-PGK-neo-FRT cassette was inserted in front of ATG codon located in the exon 2 of *Esr1* gene by homologous recombination. (b) Map of targeting vector. Constructed targeting vector in a pL253 targeting vector and linearized by *Clal* digestion is shown. (c) A representative gel image of PCR genotyping. Targeted ES cells carrying the knock-in iCre were identified by a long range PCR. Lanes 1, 18: 1 kb ladder; Lane 2, 10: without genomic DNA; lane 3, 11: wild type (wt) ES cell genomic DNA; 4–9, 12–17: genomic DNAs from targeted ES cells. (d) Positions of primer binding sites for genotyping. Primer binding sites for wild type (WT) and homozygote (*Esr1*-iCre/iCre) and heterozygote (*Esr1*-iCre/wt), and their representative PCR results are shown

FIGURE 2.

Localization of functional iCre expression in adult *Esr1*-iCre mouse organs. *Esr1*-iCre mice were crossed with Ai9 reporter line for visualization of functional iCre expression. RFP is expressed in the *Esr1*-lineage cells. RFP expression is seen in the skin (a–f), reproductive organs (g–i), brain (j, dorsal view; k, ventral view), pituitary (l), thoracic organs (m), digestive tract (n), and adrenal–kidney complex (o) of a PND60 *Esr1*-iCre mouse. Ad, adrenal gland; Cb, cerebellum; CC, cerebral cortex; Ep, epididymis; FL, forelimb; He, heart; HL, hindlimb; Ht, hypothalamus; In, intestine; Ki, kidney; Li, liver; Lu, lung; Mb, midbrain; OB, olfactory bulb; Ova, ovary; Ovi, oviduct; Po, pons; Pr, preputial gland; Sp, spleen; Te, testis; Ur, urinary bladder; Ut, uterus; Vas, Vas deferens

FIGURE 3.

Localization of functional iCre expression in embryonic mammary gland and thoracic organs. (a) RFP expression in an ED 12.5 *Esr1*-RFP mouse embryo. RFP expression is shown in the mammary bud (arrow) and developing gonad (arrow head). (b) Thoracic organ complex dissected out from an *Esr1*-lacZ mouse embryo was stained with X-gal for visualization. X-gal stained cells are seen in the developing atrium. At, atrium; Lu, lung; Th, thymus; Ve, ventricle

FIGURE 4.

Functional iCre expression in embryonic and neonatal skin and digits. Localization of functional iCre expression was visualized by X-gal staining (ED 12.5) and RFP microscopy (PND 5). X-gal stained cells are seen in the skin at ED 16.5. RFP expression is shown in the skin of PND 5 *Esr1^{iCreAi9}* mouse, but not in *Esr1^{iCre}* mouse. FL, forelimb; HL, hindlimb

FIGURE 5.

Functional iCre expression in embryonic and neonatal gonads. Localization of iCre expression was visualized by X-gal staining (ED 12.5) and RFP microscopy (ED 19.5 and PND 20). X-gal stained cells are localized to mesonephric and gonads in ED 12.5 embryo. RFP expression is seen in male and female gonads and reproductive tracts at ED 19.5 and PND20. Ad, adrenal gland; Ep, epididymis; G, gonad; He, heart; In, intestine; Ki, kidney; Li, liver; Lu, lung; M, mesonephros; Ova, ovary; Ovi, oviduct; Sp, spleen; Te, testis; Ut, uterus; Vas, vas deferens

FIGURE 6.

Functional iCre expression in embryonic brain and pituitary gland. *Esr1*-driven RFP is predominantly seen in the cerebellum, preoptic area, hypothalamus, amygdaloid cortex, and pituitary at ED 19.5. At PND 20, RFP expression is seen in cerebral cortex, preoptic area, cerebellum, hypothalamus, amygdaloid cortex, and pituitary. No RFP signal is seen in *Esr1*^{Cre} mouse. ACo, anterior cortical amygdaloid nucleus; ARC, arcuate nucleus of hypothalamus; Cb, cerebellum; CC, cerebral cortex; Ht, hypothalamus; Mb, midbrain; MPA, medial preoptic area; OB, olfactory bulb; Pit, pituitary; PLCo, posterolateral cortical amygdaloid nucleus; Po, pons; Tc, telencephalon; VMH, ventromedial hypothalamus

FIGURE 7.

Ovarian and uterine phenotypes homozygote *Esr1*-iCre mouse. (a) Gross and histological images of the reproductive organs of PND 38 mice. Note the hemorrhagic cysts and thin uteri in the *Esr1*-iCre/iCre mouse tissues. (b) Immunohistochemical detection of ESR1 expression (brown) *in uteri* of homozygous and heterozygous knock-in mice. No ESR1 expression is seen in the *Esr1*-iCre/iCre homozygous mouse uterus. AF, antral follicle; CF, cystic follicles; CL, corpus luteum; Ep, epithelium; HF, hemorrhagic follicles; Ova, ovaries; Ovi, oviducts; St, stroma; Ut, uteri

TABLE 1Summary of functional iCre expression in adult *Esr1*-iCre mice

Tissue	Relative fluorescence	Notes
Adipose Tissue	–	No fluorescence visualized.
Adrenal	++	Fluorescence is visualized in adrenal cortex.
Bladder	+++	Strong fluorescence throughout the detrusor muscle.
Blood (circulating)	–	No fluorescence seen.
Brain	++	Diffuse fluorescence throughout the cerebrum, marked increased fluorescence on the hypothalamus, fluorescence is also seen in the cerebellum. Little fluorescent signal was observed in the optic nerves and medulla oblongata.
Colon	+	Diffused and patchy fluorescence throughout mucosa.
Epididymis	+++	Strong fluorescence seen in the head, body and tail of the epididymis.
Esophagus	–	No fluorescence visualized.
Eye	++	Obvious fluorescence visible in the cornea.
Gallbladder	+	Punctate fluorescence visualized.
Heart	+	Patchy fluorescence in some of the epicardial cells.
Intestine	++	Widespread, patchy fluorescence visualized.
Kidney	+	Punctate fluorescence visualized.
Liver	+	Fluorescence visible in hepatic lobules.
Lung	+	Diffused fluorescence visualized.
Ovary	+++	Fluorescence is seen in granulosa cells, theca cells, stromal cells and surface epithelium. Not seen in oocytes.
Oviduct	+++	Fluorescence is seen in entire oviduct (infundibulum, ampulla and isthmus).
Pancreas	++	Fluorescence visualized in central region.
Penis	+++	Fluorescence is seen externally.
Pituitary	++	Prominent fluorescence is visible in the posterior pituitary, but faint fluorescence was observed in the anterior pituitary.
Preputial gland	+++	Fluorescence seen in the majority of acini.
Prostate	+++	Fluorescence is visible, most prominently in anterior prostate (coagulating gland).
Seminal vesicle	+	Weak diffused fluorescence visualized.
Skeletal muscle	+++	Obvious, strong fluorescence throughout skeletal muscle throughout the body.
Skin	+++	Strong fluorescence is visible in the epidermis throughout the body, including pads of feet and tail.
Spleen	–	No fluorescence visualized.
Testes	++	Strong fluorescence in the interstitium; punctate expression in epithelium lining the rete.
Thymus	++	Fluorescence visualized throughout the thymus.
Uterus	+++	Striated fluorescence visualized throughout the uterus.
Vagina	+	Fluorescence is visualized externally.
Vas deferens	++	Fluorescence is visualized but weaker than that in the epididymis.