

# Generation and characterization of a complete null estrogen receptor $\alpha$ mouse using Cre/LoxP technology

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**Abstract** Conventional estrogen receptor  $\alpha$  knockout (neo-ER $\alpha$ KO, neo-ER $\alpha^{-/-}$ ) mice contain a truncated and chimeric ER $\alpha$  fusion protein that retains 35% estrogen-dependent transactivation activity, and therefore the in vivo ER $\alpha$  function is difficult to study thoroughly. Furthermore, these neo-ER $\alpha^{-/-}$  mice cannot be used for tissue and temporal specific ER $\alpha$  deletion. Therefore, there is a clear need to establish a floxed ER $\alpha$  mouse line that can knockout ER $\alpha$  specifically and completely in each selected cell type. Here we generated floxed ER $\alpha$  mice using a self-excising ACN (tACE-Cre/Neo) cassette. Mating the floxed ER $\alpha$  mice with ACTB-Cre mice produces a deletion of the floxed allele disrupting the reading frame of the ER $\alpha$  transcript so that no ER $\alpha$  protein is detected in the ACTB-Cre/ER $\alpha^{-/-}$  mice. Expression of ER $\alpha$  target genes, such as G-6-PD and lactoferrin, is diminished by over 90% in the ACTB-Cre/ER $\alpha^{-/-}$  uterus, but not in the neo-ER $\alpha^{-/-}$  uterus. Furthermore, we also validated that ACTB-Cre/ER $\alpha^{-/-}$  females have a hypoplastic internal genital tract, polycystic ovaries with hemorrhagic follicles, infertility, and higher body weight. Together, our data clearly

demonstrate that the newly established floxed ER $\alpha$  mouse is a reliable mouse model for future studies of ER $\alpha$  roles in vivo in the selective estrogen target tissues. The complete knockout of ER $\alpha$  in the ACTB-Cre/ER $\alpha^{-/-}$  mice will also provide an improved mouse model to study the role of ER $\alpha$  in vivo.

**Keywords** Estrogen receptor  $\alpha$  · Transgenic animal · Female reproduction

## Introduction

Estrogens regulate the growth, differentiation, and developmental functions in a broad range of target tissues, such as the reproductive system, bone, and cardiovascular systems [1]. Estrogens actions are mediated by the cell- and tissue-specific transcriptional regulation of estrogen receptors (ERs) target genes via ERs [2]. ERs are members of the nuclear receptor (NR) superfamily and act as ligand-inducible transcription factors [3, 4], which are encoded by two distinct genes, ER $\alpha$  and ER $\beta$ . Classically, upon ligand binding, the ERs undergo a conformational change, receptor dimerization, and recruitment of distinct co-regulator complexes for transcriptional control [5]. Although in vitro assays have demonstrated that ER $\alpha$  and ER $\beta$  have similar DNA and ligand binding properties [6, 7], ER $\alpha$  and ER $\beta$  display a variety of differences in terms of tissue distribution, transcriptional activation, and knockout (KO) phenotypes [1, 6, 8, 9]. Those studies suggest that ER $\alpha$  and ER $\beta$  might regulate different target genes' expression and biological functions [2, 6, 10].

To assay the in vivo ER $\alpha$  functions, Lubahn et al. [11] generated the ER $\alpha$ KO mice (designated as neo-ER $\alpha^{-/-}$ ) via the conventional gene KO strategy by insertion of a

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Neo cassette into exon II of the mouse ER $\alpha$  gene. Neo-ER $\alpha^{-/-}$  mice do not express the full-length ER $\alpha$ , but contain a truncated and chimeric E1 protein, which results from a novel ER $\alpha$  transcript through alternative splicing, has seven amino acids in the N-terminal A/B regions replaced by the neomycin insert, and retains an intact DNA-binding domain (DBD) and ligand-binding domain (LBD). The E1 protein lacks AF-1 transactivation function, but still possesses estrogen-dependent transactivation capability; thus the conventional neo-ER $\alpha^{-/-}$  mice do not represent a complete null ER $\alpha$  mutant [12].

In order to characterize the tissue and temporal-specific ER $\alpha$  function in specific target organs, we have generated the floxed ER $\alpha$  mouse line. By mating floxed ER $\alpha$  mice with ACTB-Cre mice, which ubiquitously express Cre recombinase, we generated ER $\alpha$ KO mice (ACTB-Cre/ER $\alpha^{-/-}$ ) to validate KO efficiency and ER $\alpha$  function. Both Dupont et al. [8] and Feng et al. [13] have generated floxed ER $\alpha$  mice to target exon III of the mouse ER $\alpha$  gene. However, it has been reported that variations in the design of the floxed allele in different mouse lines might generate unique or distinct phenotypes. For example, it is expected that the floxed androgen receptor (AR) mouse line should present wild type (Wt) phenotypes with normal hormonal profile and fertility [14]; yet, there is an example of a floxed AR mouse line that has an altered hormonal profile and reduced fertility [15]. Therefore, it is necessary to establish and characterize each independent “floxed” ER $\alpha$  mouse line. The floxed ER $\alpha$  mice developed in our laboratory differ somewhat in the strategy used to “flox” exon III (see Table 1). Importantly, however, we are the first group to characterize whether any residual ER $\alpha$  protein is expressed in ER $\alpha^{-/-}$  mice (Table 1). Using different antibodies recognizing either the N terminus or C terminus of ER $\alpha$ , we demonstrate in this study the generation of a complete null ER $\alpha$  mouse without any remaining ER $\alpha$  protein. We have performed comparisons in expression of ER $\alpha$ , and of ER $\alpha$  transactivation capabilities, between the neo-ER $\alpha^{-/-}$  and the ACTB-Cre/ER $\alpha^{-/-}$  mice.

## Materials and methods

### Injection of targeted embryonic stem (ES) cells into mouse blastocysts and germline transmission of floxed ER $\alpha$

The cloned mouse ER $\alpha$  fragment was obtained from a 129Sv/J BAC clone of the ER $\alpha$  locus from the Microarray and Genomics Facility at Roswell Park Cancer Institute (Buffalo, NY) and used to generate a targeting construct where exon III of the mouse ER $\alpha$  gene was flanked by loxP sites [16]. First, an oligonucleotide containing a 34 bp loxP site was inserted into an *NheI* site 5' to exon III. Next, a self-excising ACN (tACE-Cre/Neo) cassette was inserted into an *Eco47III* site 3' to the same exon [17]. The self-excising ACN cassette includes a testis-specific promoter tACE (angiotensin-converting enzyme) driving the expression of Cre recombinase. The tACE-Cre fragment is adjacent to a Neo marker gene, and the entire tACE-Cre/Neo cassette is flanked by two loxP sites. The targeting vector was linearized and electroporated into 129 Sv/J mouse ES cells. ES cells containing the targeted ER $\alpha$  allele were identified by Southern Blot using an *EcoRV* digestion of ES cell genomic DNA. The probe consisted of a *PstII/BglIII* fragment obtained from intron II.

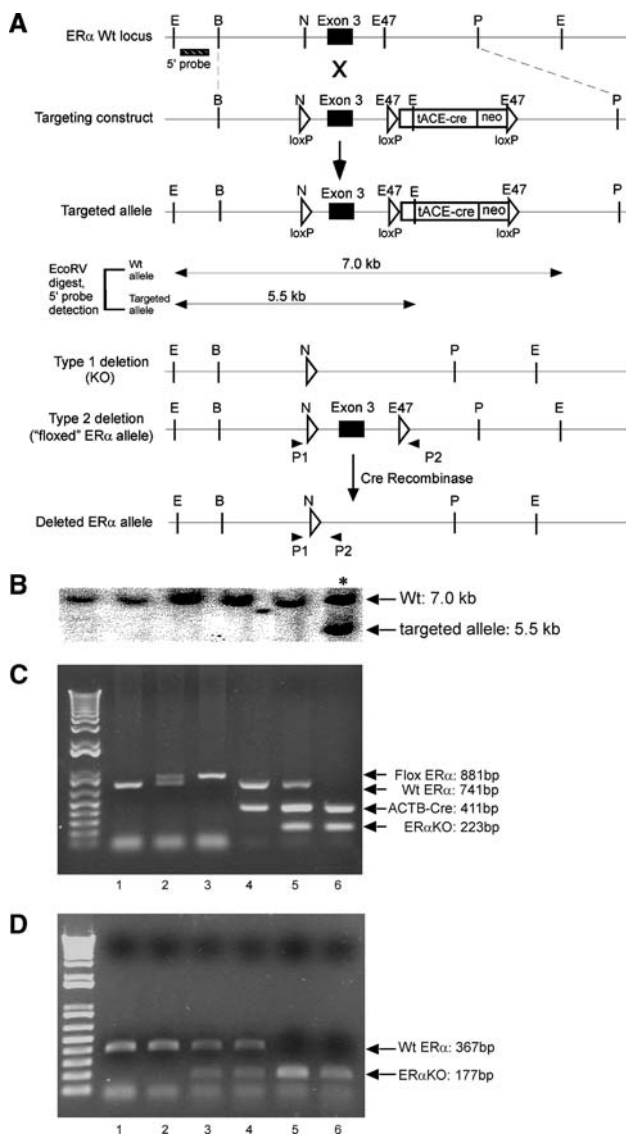
The ES cells heterozygous for the allele containing the integrated homologous recombinant were injected into Wt mouse blastocysts. Chimeric males were mated to Wt C57BL/6 females. Two types of deletions were observed to occur in the testes of chimeric males: a Type I deletion where both the ACN cassette and exon III fragments are deleted and a Type II deletion where the ACN cassette, but not exon III, is deleted. In the Type II deletion, the mutant allele is marked by two loxP sites flanking exon III of the ER $\alpha$  gene and does not contain the ACN cassette.

Southern blot analysis of tail genomic DNA obtained from the F1 progeny was performed to identify F1 mice heterozygous for the Type II deletion (ER $\alpha^{fl/+}$ ). These mice were bred to homozygosity to generate homozygote floxed ER $\alpha$  mice (ER $\alpha^{fl/fl}$ ) and backcrossed with the pure C57BL/

**Table 1** Comparison of currently available floxed ER $\alpha$  transgenic mice with the conventional neo-ER $\alpha^{-/-}$  mice

	Targeted ER $\alpha$ exon	Cassette for ES selection	Neo cassette removal	Residual ER $\alpha$ protein detected	ER $\alpha$ target gene expression in uterus
Couse et al. [1]	Exon II	PGK-neo	(knock-in)	+ E1 protein; + immunoreactive ER $\alpha$ in uterus	Partially reduced
Dupont et al. [8]	Exon III	TK-neo	In vivo excision	– C terminus; NR* N terminus	NR*
Feng et al. [13]	Exon III	PGK-neo/HSV-TK	In vitro excision	NR*	NR*
Chen and Wolfe et al.	Exon III	tACE-Cre/Neo	In vivo excision	– N terminus (H-184); – C terminus (MC-20)	Over 90% diminished

\* NR: data not reported



**Fig. 1** Targeted disruption of mouse ER $\alpha$  gene using Cre-loxP strategy. **a** Schematic map of Wt ER $\alpha$ , targeting construct, targeted allele, Type 1 deletion, Type 2 deletion, and deleted ER $\alpha$  allele after cre-recombination. *NheI* and *Eco47III* sites were used to insert the loxP sequence and pACN cassette. The primer set: P1 and P2, was used for genotyping. E (*EcoRV*), B (*BamHI*), N (*NheI*), E47 (*Eco47III*), P (*PstI*). **b** Southern Blot analysis was used to screen ES cell clones using the 1.5 kb 5' probe. Southern Blot shows the positive clone (\*) and five Wt clones. **c** Genotyping of different ER $\alpha$  mutant mice using primer mixture: P1, P2 and *Cre* primers. Lane 1, Wt mice (ER $\alpha$ <sup>+/+</sup>); Lane 2, floxed ER $\alpha$  heterozygote mice (ER $\alpha$ <sup>fl/+</sup>), the size of floxed ER $\alpha$  is 140 bp more than that of Wt ER $\alpha$ ; Lane 3, floxed ER $\alpha$  homozygote mice (ER $\alpha$ <sup>fl/fl</sup>); Lane 4, ACTB-Cre transgenic mice (ACTB-cre/ER $\alpha$ <sup>+/+</sup>), the size of Cre fragment was 411 bp; Lane 5, heterozygote ER $\alpha$ KO mice (ACTB-Cre/ER $\alpha$ <sup>+/+</sup>), in which the size of floxed ER $\alpha$  allele was reduced to 223 bp after the deletion of intervening DNA via ACTB-cre recombinase; Lane 6, homozygote ER $\alpha$ KO mice (ACTB-Cre/ER $\alpha$ <sup>-/-</sup>), in which the size of both floxed ER $\alpha$  alleles was reduced to 223 bp by ACTB-cre recombinase and only deleted ER $\alpha$  band was present. **d** Semi-quantitative RT-PCR using primer sets P3 and P4 to detect the presence of ER $\alpha$  transcripts in the uterine cDNA from Wt, ACTB-Cre/ER $\alpha$ <sup>+/-</sup>, or ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice. Wt: Lanes 1 and 2; ACTB-Cre/ER $\alpha$ <sup>+/-</sup>: Lanes 3 and 4; ACTB-Cre/ER $\alpha$ <sup>-/-</sup>: Lanes 5 and 6

6 strain to generate a more homogenous background for further experimental analyses. All the animals were fed using a standard diet (#5001, LabDiets, Richmond, IN) and all animal procedures were approved by the animal care and use committee of the University of Rochester Medical Center, in accordance with National Institutes of Health guidelines.

#### Generation and genotyping of ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice

In order to generate the ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice, the ER $\alpha$ <sup>fl/fl</sup> female mice were crossed with ACTB-Cre transgenic male mice (Jackson Laboratories, Bar Harbor, ME). These mice ubiquitously express *Cre* recombinase under the control of the  $\beta$ -actin promoter. After two generations of mating, ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice were developed. Genomic DNA was isolated from tail biopsies and used as a template for PCR genotyping as previously described [14]. The primer pairs used were as follows: P1: 5'-AGGC TTTGTCTCGCTTTCC-3', P2: 5'-GATCATT CAGAGAG ACAAGAGGAACC-3' (see Fig. 1a; floxed ER allele). *Cre*: forward, 5'-AGGTGTAGAGAAGGCACTTAGC-3'; reverse, 5'-CTAATCGCCATCTTCCAGCAGG-3'. The sizes of the P1–P2 fragments from Wt ER $\alpha$ , floxed ER $\alpha$ , and ER $\alpha$  KO allele were 741, 881, and 223 bp, respectively. The size of the *Cre* fragment was 411 bp. The PCR for the genotyping was performed with the P1, P2, and *Cre* primer mixture.

#### Fertility test

Five 14- to 16-week-old Wt, ACTB-Cre/ER $\alpha$ <sup>+/-</sup>, and ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females were mated with known fertile males for 18 weeks. The number of pups and litters was recorded.

#### RNA isolation, semi-quantitative RT-PCR, and real-time PCR

Total RNA was extracted and purified from the uteri of different female genotypes at 12 weeks of age using Trizol (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions, then 3  $\mu$ g RNA was subjected to reverse transcription using Superscript III (Invitrogen, Carlsbad, CA). The resulting first strand cDNA was subjected to semi-quantitative RT-PCR with the following ER $\alpha$ -specific primer pairs: P3, 5'-GGTGCCCTACTA CCTGGAG-3' and P4, 5'-GCCCACTTCGT AACACT TGCG C-3'. P3 and P4 are located in the mouse ER $\alpha$  exon II and exon IV respectively. The sizes of P3–P4 fragments from Wt and ER $\alpha$ KO transcripts were 367 and 177 bp, respectively. The real-time PCR was performed with first

strand cDNA, specific gene primers, and SYBR Green PCR Master Mix (Biorad, Hercules, CA). The PCR cycle was performed as follows: 94°C for 3 min, 40 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 30 s on an iCycler iQ Multi-color real-time PCR detection system (Biorad, Hercules, CA). Primer sequences were as follows: G-6-PD: sense, 5'-TTAAAGCCACTCCAGAAGAAAGAC-3'; antisense, 5'-TCCACGATGATGCGGTTCC-3'. Lactoferrin: sense, 5'-TAGCAGCAGTTAGAAGAGAAGATG-3'; antisense, 5'-GGGCACAGAGATTGGATTTGG-3'. 18S: sense, 5'-TGCCTTCCTTGGATGTGGTAG-3'; antisense, 5'-CGTCTGCCCTATCAACTTTCG-3'. Each sample was run in triplicate, and data were analyzed using iCycler iQ software (Biorad, Hercules, CA).

### Antibodies

Mouse monoclonal anti-GAPDH antibody 6C5, rabbit polyclonal antibody H-184 directed against the 2–185 amino acids (a.a.) of human ER $\alpha$  N terminus, and MC-20 against the last 20 a.a. of the mouse ER $\alpha$  C terminus were obtained from Santa Cruz Biotechnology.

### ER $\alpha$ KO N terminus cDNA construct

The putative N-terminal ER $\alpha$ KO cDNAs were generated by PCR from the pcDNA3-flag-mER $\alpha$  plasmid using the forward primer: 5'-CGGGATCCATGACCATGACCCCTCACACCAAAG-3' and reverse primer: 5'-CGGAATTC TCATTGTGTCCTGTAGAAGGCGGGAG-3' and subsequently constructed into pcDNA3-flag vector. The correct construction of the plasmid was verified by sequencing and protein expression was confirmed by TNT in vitro expression.

### Western blotting

Protein extracts were prepared from the uteri of different mouse genotypes as described previously [18, 19], 80  $\mu$ g of protein was separated on a 10% gel by SDS-PAGE, and transferred onto a nitrocellulose membrane. ER $\alpha$  protein was detected with the rabbit polyclonal antibodies H-184 and MC-20 (Santa Cruz, CA). The same blot was hybridized with an anti-GAPDH antibody to control for equal loading (Santa Cruz, CA).

### Immunohistochemistry (IHC)

The IHC was carried out as described previously [19, 20]. In brief, the paraffin-embedded tissue blocks were cut at 4- $\mu$ m thickness, dewaxed, and rehydrated. Antigens were retrieved by boiling in 10 mM citrate buffer (pH 7.0) for

10 min. The sections were incubated in 0.5% H<sub>2</sub>O<sub>2</sub> in PBS for 30 min at room temperature to quench endogenous peroxidase. To block nonspecific binding, sections were incubated in 5% normal serum prepared from the host of secondary antibodies for 1 h at 4°C. Sections were incubated with ER $\alpha$  antibodies (MC-20, 1:400) in 3% BSA in PBS overnight at 4°C, and then incubated with 1:200 diluted biotinylated secondary antibody (Vector Laboratories) and ABC solution (Vector Laboratories). The tissues were then stained by AEC followed by Mayer's hematoxylin counterstaining (DAKO, Carpinteria, CA). Negative controls were incubated without primary antibody.

## Results and discussions

### Generation of floxed ER $\alpha$ mouse

Exon III of the ER $\alpha$  gene encodes the first zinc finger of the ER $\alpha$ -DBD and plays an important role in ER-ERE binding and transcriptional regulation of ER $\alpha$  target genes [1]. The deletion of exon III results in a translational frameshift in ER $\alpha$  mRNA by the splicing of exon II and IV; thus, we generated a floxed ER $\alpha$  mouse line by targeting exon III. Homologous recombination was used to insert a floxed ER $\alpha$  allele into the ES cell genomic DNA. A self-excising neomycin cassette was used to select for targeting vector incorporation. The strategy is outlined in Fig. 1a. Homologous recombination was determined by Southern Blot of an *EcoRV*-digested ES cell genomic DNA (Fig. 1b), using the probe consisting of a *PstI/BglII* fragment obtained from intron II (Probe, Fig. 1a). Targeted ES cells were injected into C57BL/6 blastocysts. The self-excising tACE-Cre/Neo cassette was then removed from the mutant allele in vivo. Retaining a Neo cassette in vivo was shown to have unpredictable consequences on the phenotype of mice, including the generation of a hypomorphic allele with a wide range of phenotypic abnormalities [21, 22], and mis-regulation of adjacent genes [23]. The tACE (angiotensin-converting enzyme) promoter present in the ACN cassette has been shown to specifically activate *Cre* recombinase activity in the testis sperm cells, but not in other tissues [17]. Thus, self-excision of the ACN cassette in the sperm of chimeric male mice resulted in transmitting the mutant allele that contained the floxed ER $\alpha$  gene, lacking the ACN cassette, to the F1 progeny. F1 mice heterozygous for the Type II deletion were selected (heterozygote floxed ER $\alpha$  mice, ER $\alpha$ <sup>fl/+</sup>) via Southern blot and PCR analysis of tail DNA and bred to generate homozygous floxed ER $\alpha$  mice (ER $\alpha$ <sup>fl/fl</sup>). Table 1 includes the targeting strategy used by our laboratory and by two other groups.

### Generation of ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice

The ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice were generated by mating the ER $\alpha$ <sup>fl/fl</sup> female mice with ACTB-cre transgenic male mice. PCR performed using the *Cre* primers, and P1 and P2 primers, was able to distinguish the Wt mice (ER $\alpha$ <sup>+/+</sup>, 741 bp band), heterozygote floxed ER $\alpha$  mice (ER $\alpha$ <sup>fl/+</sup>; 741 bp and 881 bp band), homozygote floxed ER $\alpha$  mice (ER $\alpha$ <sup>fl/fl</sup>; only 881 bp band), ACTB-cre transgenic mice (ACTB-Cre/ER $\alpha$ <sup>+/+</sup>; 411 and 741 bp band), heterozygous ER $\alpha$ KO mice (ACTB-Cre/ER $\alpha$ <sup>+/-</sup>; 411, 741, and 223 bp band), and homozygous ER $\alpha$ KO mice (ACTB-Cre/ER $\alpha$ <sup>-/-</sup>; 411 and 223 bp) (Fig. 1c, lanes 1–6, respectively). PCR products were cloned and further sequenced to confirm that the targeted fragment contained the predicted sequence (data not shown). The disruption of ER $\alpha$  gene expression was also confirmed by semi-quantitative RT-PCR of messenger RNA from the uterus. Using the primer set of P3 and P4 located in exon II and IV, a PCR product of 177 bp was amplified from reverse transcribed cDNA of ACTB-cre/ER $\alpha$ <sup>-/-</sup> uterus. Reverse transcribed cDNA of Wt mouse uterus produced a 367 bp product, consistent with the expected size of the Wt locus, while ACTB-cre/ER $\alpha$ <sup>+/-</sup> heterozygous mice reveal the presence of a Wt (367 bp) and KO (177 bp) transcript (Fig. 1d). DNA sequencing showed that the KO transcripts completely lack exon III (data not shown).

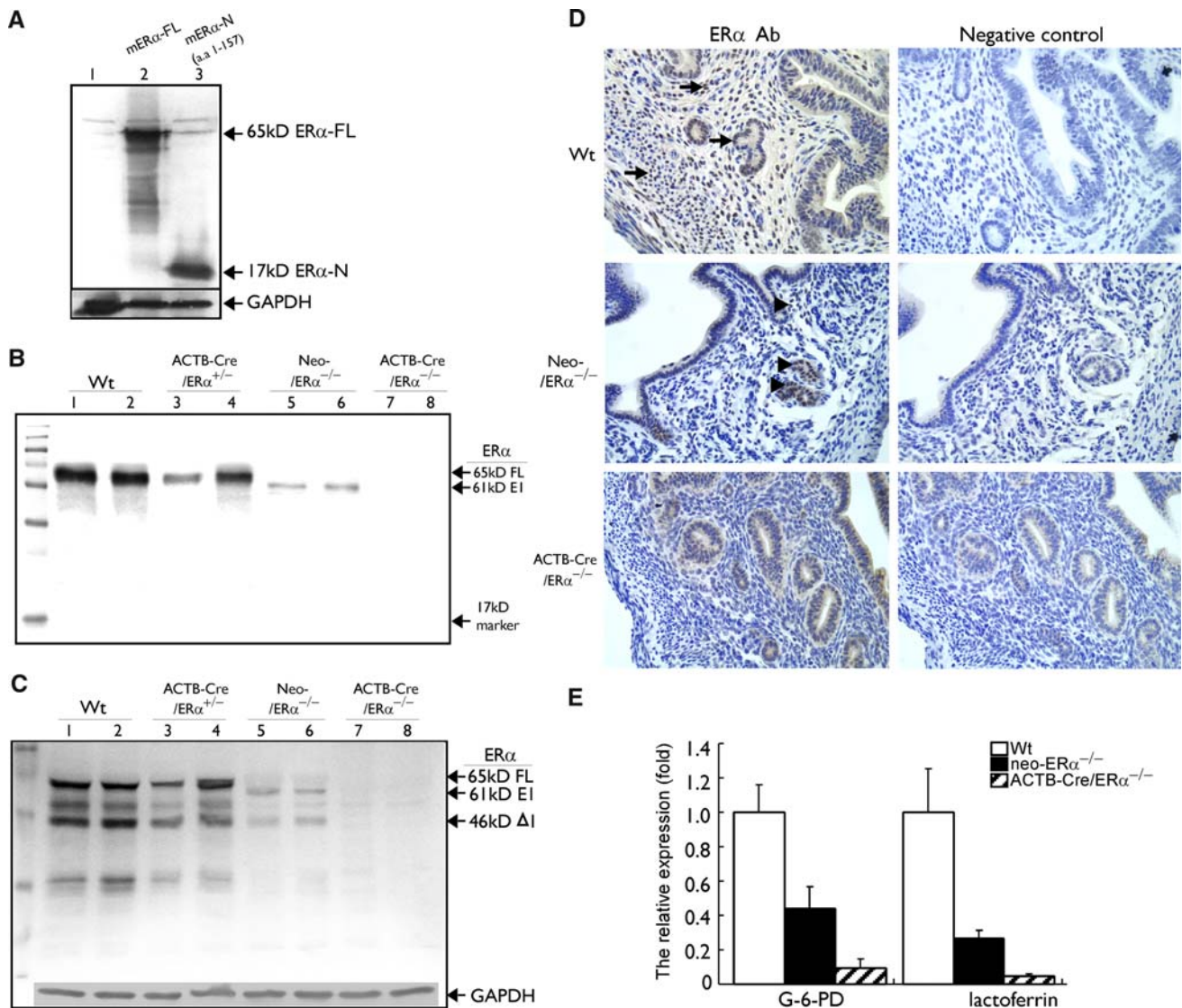
Further confirmation of the complete null ER $\alpha$  mutant in the ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice

Deletion of exon III by Cre-mediated recombination resulted in a premature stop codon at the new codon 158 position due to the splicing of exon II and IV. Thus, the putative truncated ER $\alpha$  protein from ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice would be predicted to be 157 a.a. in length in the absence of a.a. from the ER $\alpha$  DBD and LBD. To examine whether the putative mouse ER $\alpha$  N polypeptide (a.a. 1–157) is expressed in the ACTB-Cre/ER $\alpha$ <sup>-/-</sup> uterine extracts, we first validated which ER $\alpha$  antibody can recognize mouse ER $\alpha$  N terminus. A rabbit polyclonal antibody, H184, was originally generated to recognize the N terminus of human ER $\alpha$  (a.a. 2–185). Previous studies have shown that this antibody could recognize the mouse ER $\alpha$  [24]; however, the immunoreactive epitopes of H184 are not well characterized. It was unclear whether H184 could recognize the first 157 a.a. of mouse ER $\alpha$ . Therefore, the mouse ER $\alpha$  N terminus (a.a. 1–157) was cloned and expressed in Cos-1 cells. The Cos-1 cell lysates were subjected to western blot analysis using the H184 antibody, and our results indicate that this antibody could detect both mouse ER $\alpha$  full-length and N-terminal polypeptide (a.a. 1–157) (Fig. 2a, lanes 2 and 3).

We then used western blot analysis to examine whether ER $\alpha$  N terminus was expressed in ACTB-Cre/ER $\alpha$ <sup>-/-</sup> uterine extracts using the H184 antibody. The Wt and neo-ER $\alpha$ <sup>-/-</sup> mice revealed a 65-kD ER $\alpha$  protein and a novel 61-kD E1 protein, respectively (Fig. 2b, lanes 1, 2 and 5, 6). In contrast, the disruption of ER $\alpha$  in our ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice resulted in the absence of the putative ER $\alpha$  N-terminal 157-amino-acid polypeptide (Fig. 2b, lanes 7 and 8). The deletion of ER $\alpha$  exon III in the ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice produces a nonsense mutation that results in a premature termination codon in ER $\alpha$  exon IV. The studies in the eukaryotic cells have shown that the nonsense mutation may lead to mRNA decay and prevent the accumulation of truncated protein [25]. Although it is expected that the premature termination codon in the ER $\alpha$  exon IV will yield a 157-amino-acid N-terminal ER $\alpha$  polypeptide, nonsense-mediated mRNA decay (NMD) could degrade the mutant ER $\alpha$  mRNAs containing the premature termination codon and prevent the synthesis of the 157-amino-acid N-terminal ER $\alpha$  polypeptide in the ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice. It is also possible that the ER $\alpha$  N terminus is present, but undetectable, in ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice, due to instability of the protein. To our knowledge, this is the first report to definitely show the absence of the ER $\alpha$  N terminus in ER $\alpha$ KO mice.

Moreover, using the MC-20 antibody, recognizing the ER $\alpha$  C terminus, western blotting of Wt uterine extracts revealed not only a 65-kD product, consistent with full-length ER $\alpha$ , but also a 46-kD product ER $\alpha$   $\Delta$ 1. This is an isoform of ER $\alpha$  splicing of exon II and encodes an in-frame ER $\alpha$  protein with intact DBD and LBD domains by utilizing the start codon in exon III and a favorable Kozak sequence (Fig. 2c, lanes 1 and 2) [26]. The ER $\alpha$   $\Delta$ 1 protein was found to be expressed in the mouse uterus and human cells [27, 28]. In the previously generated neo-ER $\alpha$ <sup>-/-</sup> mice, a 65-kD full-length ER $\alpha$  protein was not detectable, but a 61-kD-truncated E1 protein was detected in the uterine extracts of neo-ER $\alpha$ <sup>-/-</sup> mice, which was consistent with the results detected by ER $\alpha$  antibody H-184 (Fig. 2c, lanes 5 and 6). In addition, a 46-kD ER $\alpha$   $\Delta$ 1 was detected in the uterine extracts of neo-ER $\alpha$ <sup>-/-</sup> mice (Fig. 2c, lanes 5 and 6). Our ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice did not express ER $\alpha$   $\Delta$ 1 due to the deletion of exon III. Collectively, the full disruption of ER $\alpha$  in our ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice was shown by the absence of any ER $\alpha$  residual protein immunoreactivity with either the ER $\alpha$ -N-terminal antibody (H-184) or ER $\alpha$ -C-terminal antibody (MC-20) in the uterine extracts (Fig. 2b, c).

In addition, we examined ER $\alpha$  expression in the paraffin-embedded uterine tissue by IHC staining. ER $\alpha$  was mainly expressed in the uterine stromal and glandular epithelial cells with little positive nuclear staining in the luminal epithelial cells of 3-month-old Wt mice (Fig. 2d,



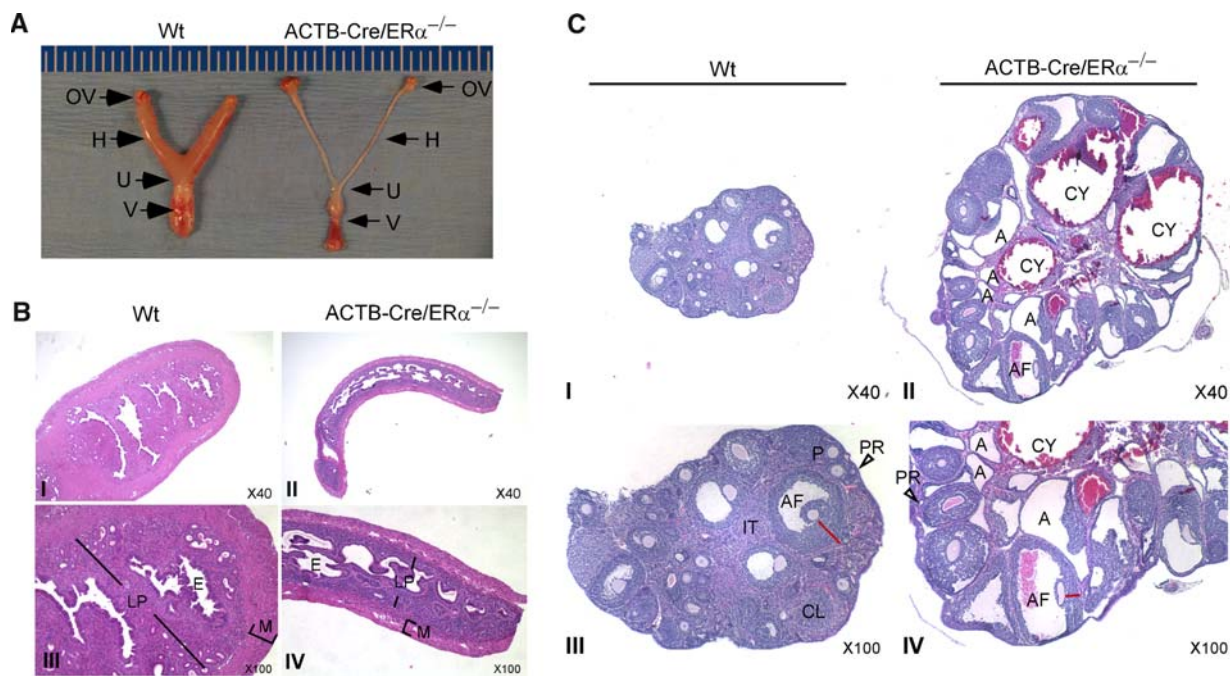
**Fig. 2** Detection of the expression of ER $\alpha$  protein and estrogen target genes to prove the complete deletion of ER $\alpha$  in the ACTB-Cre/ER $\alpha^{-/-}$  mice. **a** The recognition of mouse ER $\alpha$  N terminus by the ER $\alpha$  antibody (H-184). The full length and N terminus of mouse ER $\alpha$  were cloned into pCDNA3 plasmids and transfected into Cos-1 cells for the western blot analysis. Our results indicated that both mouse ER $\alpha$  full length and N-terminal (a.a. 1–157) proteins could be recognized by ER $\alpha$  antibody H-184. Note that the H-184 antibody was originally produced against the human ER $\alpha$ . **b–c** Western blot analysis of ER $\alpha$  protein in the uterus of Wt, neo-ER $\alpha^{-/-}$ , ACTB-Cre/ER $\alpha^{+/+}$ , and ACTB-Cre/ER $\alpha^{-/-}$  using antibody H-184 against mouse ER $\alpha$  N terminus (**b**) or antibody MC-20 against the C terminus (**c**). Note that neo-ER $\alpha^{-/-}$  uteri encode a 61-kD chimeric protein (**b** and **c**, Lanes 5 and 6) and a 46-kD ER $\alpha$   $\Delta$ 1 protein (**c**, Lanes 5 and 6). No ER $\alpha$  immunoreactivity was detected in ACTB-Cre/ER $\alpha^{-/-}$  uteri (**b** and **c**, Lanes 7 and 8). GAPDH was immunoblotted as an

arrows). The majority of positive ER $\alpha$  staining in the stromal cells was abolished in the neo-ER $\alpha^{-/-}$  uterus, however, the positive nuclear ER $\alpha$  staining can still be found in some glandular epithelial and stromal cells (Fig. 2d, arrowheads). In contrast, there was no positive

indication of equal loading. **d** IHC analyses of ER $\alpha$  protein in the Wt, neo-ER $\alpha^{-/-}$ , and ACTB-Cre/ER $\alpha^{-/-}$  uterus. MC-20 antibody was used to locate ER $\alpha$  in paraffin-embedded tissue. ER $\alpha$  was mainly expressed in the nuclei of myometrium and glandular epithelial cells (arrow) in the Wt uterus. Note that positive nuclear ER $\alpha$  staining can still be found in the nuclei of some glandular epithelial and stromal cells (arrowhead) in the neo-ER $\alpha^{-/-}$  mice; no positive nuclear ER $\alpha$  staining was detected in our newly generated ACTB-Cre/ER $\alpha^{-/-}$  mice. **e** The comparison of the expression levels of the estrogen target genes among Wt, neo-ER $\alpha^{-/-}$ , and ACTB-Cre/ER $\alpha^{-/-}$  by real-time PCR. Assays were performed on RNA from the uterus of each individual mouse and then averaged for the gene expression. 18S acted as internal control. Note that the expressions of G-6-PD and lactoferrin were shown more dramatically decreased in the ACTB-Cre/ER $\alpha^{-/-}$  uterus

nuclear ER $\alpha$  staining, which could be detected in the ACTB-Cre/ER $\alpha^{-/-}$  mice, except some diffuse cytoplasmic background staining (Fig. 2d).

Real-time PCR analysis was used to detect the transcription of estrogen target genes in the mouse uterine tissue.



**Fig. 3** Comparison of uterus and ovary of 16-week-old Wt and ACTB-Cre/ER $\alpha^{-/-}$ . **a** Ventral views of the body of the uterus (U), uterine horns (H), ovaries (OV), and vagina (V). **b** Histological analyses of mouse uterus. Note that ACTB-Cre/ER $\alpha^{-/-}$  uterus was much thinner with a substantial reduction in the total number of stromal cells in the myometrium and lamina propria. M, myometrium;

LP, lamina propria, E, epithelia. **c** Histological analyses of the ovary. Note that ovaries from adult ACTB-Cre/ER $\alpha^{-/-}$  mice (16-week-old) displayed dilated, frequently hemorrhagic cysts (CY); an excess of atretic follicles (A); normal primordial (PR), primary (P) and antral follicles (AF) with few interstitial compartments (IT); and lack of corpus luteum (CL)

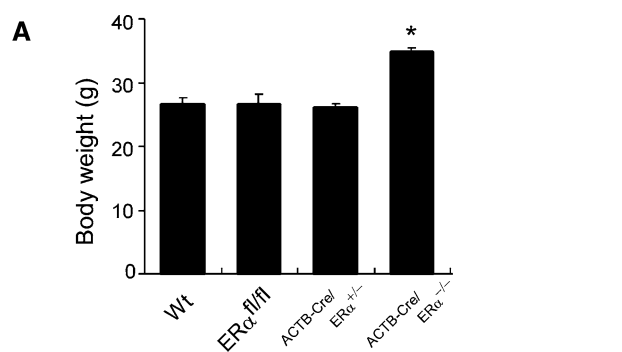
Compared to the Wt mice, the expressions of G-6-PD and lactoferrin in neo-ER $\alpha^{-/-}$  uterus were  $0.44 \pm 0.125$  and  $0.265 \pm 0.057$ , respectively, whereas, the expressions of G-6-PD and lactoferrin in the ACTB-Cre/ER $\alpha^{-/-}$  uterus were dramatically decreased to  $0.093 \pm 0.051$  and  $0.050 \pm 0.012$ , respectively (Fig. 2e). Collectively, these results demonstrate that our newly generated ACTB-Cre/ER $\alpha^{-/-}$  mice are indeed a complete ER $\alpha$  null mutant and provide a mouse model completely lacking in ER $\alpha$  signaling to study the role of ER $\alpha$  in vivo. Furthermore, direct comparisons with the neo-ER $\alpha^{-/-}$  mouse underscore the value of analyzing the ACTB-Cre/ER $\alpha^{-/-}$  mice as a model of ER $\alpha$  deficiency.

#### Defective reproductive system in ACTB-Cre/ER $\alpha^{-/-}$ females

To confirm the phenotypes that have been reported in other ER $\alpha$ KO mouse models, more than five age-matched littermates were used for each experiment. ACTB-Cre/ER $\alpha^{-/-}$  females had similar appearing external genitalia as Wt females; however, the vagina of ACTB-Cre/ER $\alpha^{-/-}$  females did not display any cyclic changes when vaginal smears were performed (data not shown). Gross internal anatomical examination revealed that ACTB-Cre/ER $\alpha^{-/-}$  uterus and vagina were hypoplastic and presented a much

more severe uterine phenotype than the hypomorphic phenotype (half the weight of Wt littermates) in the neo-ER $\alpha^{-/-}$  uterus (Fig. 3a) [1]. Histological analyses showed that the ACTB-Cre/ER $\alpha^{-/-}$  uterus contained substantially reduced numbers of stromal cells in both the myometrium and lamina propria, which resulted in significant differences in the uterine diameter between Wt and ACTB-Cre/ER $\alpha^{-/-}$  females, however, the length of uterus had no significant difference between Wt and ACTB-Cre/ER $\alpha^{-/-}$  females (Fig. 3b, I vs. II; III vs. IV), which indicate that the growth of uteri along the body axis is independent of ER $\alpha$  function. This finding agrees with other ER $\alpha$ KO models [8].

As shown in Fig. 3c, in the ovary of Wt mice, primordial follicles (PR, *arrowhead*), primary follicles (P), antral follicles (AF), and corpus luteum (CL) were all present and well organized, and the interstitial compartment (IT) was abundant and consisted of compact clusters of glandular and fibroblast cells. In contrast, ovaries from adult ACTB-Cre/ER $\alpha^{-/-}$  females displayed many large dilated, hemorrhagic cysts (CY). The primordial follicles (PR, *arrowhead*) and primary follicles were observed in the ACTB-Cre/ER $\alpha^{-/-}$  ovary, but with greatly reduced numbers compared to Wt mice. The antral follicles were also observed in ACTB-Cre/ER $\alpha^{-/-}$  ovaries (AF); however, the thickness of the granulosa cells in the antral follicles of ACTB-Cre/ER $\alpha^{-/-}$  females was significantly reduced (red



### B Fertility test of ER $\alpha$ mutant females

Genotype	n	litters	pups	pups per litter	litters per female
Wt	5	18	161	8.94 $\pm$ 0.87	3.60 $\pm$ 0.55
ACTB-Cre/ER $\alpha$ +/+	5	19	164	8.63 $\pm$ 0.83	3.80 $\pm$ 0.45
ACTB-Cre/ER $\alpha$ -/-	5	0	0	0	0

14- to 16-week-old females were bred with known fertile males for 18 weeks. Results are presented as means  $\pm$  SD.

**Fig. 4** Body weight and fertility of ER $\alpha$  mutant females. **a** Body weights of Wt, ER $\alpha$ <sup>fl/fl</sup>, ACTB-Cre/ER $\alpha$ <sup>+/+</sup>, and ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females. \* $P < 0.05$ ; ACTB-Cre/ER $\alpha$ <sup>-/-</sup> versus the other females. Values are presented as mean  $\pm$  SEM. ( $n = 8$ ). **b** ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females are infertile after 18 weeks of fertility test as compared with the normal fertility of ACTB-Cre/ER $\alpha$ <sup>+/+</sup> and Wt females

line, III vs. IV). More prominently, ACTB-Cre/ER $\alpha$ <sup>-/-</sup> ovaries had few interstitial compartments, but an excess of atretic follicles which were derived from the apoptotic antral follicles due to their inability to develop to the pre-ovulatory stage (A). No corpus luteum was observed in ACTB-Cre/ER $\alpha$ <sup>-/-</sup> ovaries. Overall, folliculogenesis in ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females is defective and can only progress into the antral stage. These findings agree with previous studies showing that a functional ER $\alpha$  is required for the functional maturation of preovulatory follicles [8, 29]. Furthermore, our results also suggest that ER $\alpha$  is necessary for interstitial glandular cell development.

### Increased body weight and infertility of ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females

The analyses of ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females showed that they developed obesity: the body weight of young adult ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females (12-week-old) was significantly higher than the control mice ( $P < 0.05$ , Fig. 4a). ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females had significant lipid accumulation in the subcutaneous white adipose tissues (WATs) (data not shown). Those results agree with other studies that have examined the increased body weight of ovariectomized mice and neo-ER $\alpha$ <sup>-/-</sup> females, as well as the role of ER $\alpha$  in suppressing the development of WATs [30, 31]. In agreement with previously published results [8, 11],

ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females were infertile as shown by 18 weeks of continuous mating test (Fig. 4b). Fertility of Wt and ACTB-Cre/ER $\alpha$ <sup>+/+</sup> females was normal and comparable.

In conclusion, we have generated floxed ER $\alpha$  mice using a self-excising ACN (tACE-Cre/Neo) cassette. Although both Dupont et al. [8] and Feng et al. [13] have generated floxed ER $\alpha$  mice, our floxed ER $\alpha$  mice were developed using a different targeting construct strategy (Table 1). It is true that this mouse model would be one of three reporting a floxed ER $\alpha$  locus, however, ours is the only study that actually compares the effect of an in vivo deletion of exon III with the insertional mutagenesis used to produce the neo-ER $\alpha$ <sup>-/-</sup> mouse. We show a much more severe uterine phenotype when exon III is deleted in the ACTB-Cre/ER $\alpha$ <sup>-/-</sup> females than the hypomorphic phenotype in neo-ER $\alpha$ <sup>-/-</sup> females [1]. Furthermore, we characterized any residual ER $\alpha$  protein being expressed in ER $\alpha$ KO mice (Table 1). We showed in mouse uterine extracts that our ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice lack any detectable ER $\alpha$  protein versus a truncated protein in the neo-ER $\alpha$ <sup>-/-</sup> line using antibodies recognizing either the N-terminus or C-terminus of ER $\alpha$ . To our knowledge, this is the first report to definitely show the absence of the ER $\alpha$  N terminus in ER $\alpha$ KO mice. We also showed that there are almost completely abolished expressions of ER $\alpha$  target genes, G-6-PD and lactoferrin, in the ACTB-Cre/ER $\alpha$ <sup>-/-</sup> uterus, whereas expression of these genes was still 44% and 27% of Wt values, respectively, in the neo-ER $\alpha$ <sup>-/-</sup> uterus. Using our ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mouse model, we have characterized the function of ER $\alpha$  in the female genital tracts. These studies form the basis for the use of the floxed ER $\alpha$  mouse to selectively and completely delete the ER $\alpha$  gene in a tissue- or temporal-specific manner by breeding with transgenic mice harboring promoter specific Cre. The complete knockout of ER $\alpha$  in the ACTB-Cre/ER $\alpha$ <sup>-/-</sup> mice also provides an improved mouse model to study the role of ER $\alpha$  in vivo.

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