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Generation of ERα-floxed and knockout mice using the Cre/LoxP system

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ABSTRACT

Estrogen receptor alpha (ERα) is a nuclear receptor that regulates a range of physiological processes in response to estrogens. In order to study its biological role, we generated a floxed ERα mouse line that can be used to knock out ERα in selected tissues by using the Cre/LoxP system. In this study, we established a new ERα knockout mouse line by crossing the floxed ERα mice with Cre *deleter* mice. Here we show that genetic disruption of the ERα gene in all tissues results in sterility in both male and female mice. Histological examination of uterus and ovaries revealed a dramatically atrophic uterus and hemorrhagic cysts in the ovary. These results suggest that infertility in female mice is the result of functional defects of the reproductive tract. Moreover, female knockout mice are hyperglycemic, develop obesity and at the age of 4 months the body weight of these mice was more than 20% higher compared to wild type littermates and this difference increased over time. Our results demonstrate that ERα is necessary for reproductive tract development and has important functions as a regulator of metabolism in females.

1. Introduction

Estrogens are sex steroids that regulate a variety of physiological processes including growth, differentiation and function of reproductive tissues. Sex steroids also have important roles in non reproductive tissues [1], including regulation of metabolism and in the cardiovascular system. The biological actions of estrogens are mediated by the estrogen receptors, ERα (NR3A1) and ERβ (NR3A2), which are ligand regulated transcription factors belonging to the nuclear receptor family [2]. This family has a typical structural architecture with an N-terminal transactivation domain, a zinc finger type DNA binding domain (DBD) and a C-terminal ligand binding domain (LBD) that confers dimerization and interactions with coregulators. The mouse ERα gene spans over more than 220 kb and is located on chromosome 10 [3]. It contains at least nine exons with the start codon in exon 2, the sequence coding for the first zinc-finger in the DBD in exon 3 and the LBD in exons 6–9 [4].

ERα and ERβ bind to similar sequences in promoters and enhancers and both receptors are activated by estrogens but the receptors are often expressed in different cells and are also believed to have unique target genes [5]. One powerful method to analyze functions of these receptors *in vivo* is to use gene targeting to introduce specific mutations in the genes. Several estrogen receptor knockout mouse models have been developed to study ERα functions. The first described model was generated using a conventional strategy of inserting a neo gene in exon 2 of the ERα gene [6]. In this study female mice were reported to be infertile with hypoplastic uteri and hyperemic ovaries. This knockout mouse line was later shown to have residual ERα activity [7]. Recent strategies to develop ERα knockout mouse lines have employed the Cre/loxP system producing deletions of targeted regions in the genome [8–12]. The Cre/loxP system can also be used to generate tissue specific knockouts. In this case floxed mice are crossed with transgenic Cre mice to produce a deletion in those cells where the Cre transgene is expressed.

To investigate the biological role of ERα *in vivo* we generated floxed ERα mice for cell specific deletion of the ERα gene. Our strategy was to target exon 3 which encodes the first zinc finger of the DBD of ERα. Deletion of this exon causes a frame shift in the coding region that introduces a stop codon in exon 4 so that the predicted expressed protein would neither contain DBD nor LBD and thus have no remaining ERα activity. In this study we have crossed the floxed ERα mice with Cre *deleter* mice to generate ERα knockout mice with a deletion of ERα in all cells in the body. We show that both male and female mice lacking functional ERα are infertile. Female mice have severe defects in the uterus and ovaries which are likely to explain the infertility. In addition female ERα knockout mice have increased basal blood glucose levels and become obese which supports the notion that ERα is an important regulator of both reproduction and metabolism.

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2. Materials and methods

2.1. Generation of ERα-floxed and null mutant mice

A genomic BAC clone from the mouse ERα locus was isolated from a 129/SvJ library by Incyte Genomics using primers corresponding to exon 3. An 8 kb EcoRV and a 10 kb BamHI fragment from this clone were subcloned into pBS-KS (Stratagene) and used to make the targeting construct. Briefly, a loxP site was cloned into the NheI site 5′ of exon 3 and a fragment containing a loxP site and an FRT flanked neo cassette was cloned into the Eco47III site 3′ of exon 3. The targeting construct was linearized and electroporated into RW4 129SvJ ES cells and selected with G418 on embryonic fibroblast feeder cells. Resistant clones were analyzed by Southern blot analysis after BamHI digestion with a 3′ external probe (HpaI/BamHI fragment) as illustrated in Fig. 1A. ES cell lines exhibiting homologous recombination were injected into C57BL/6J blastocysts that were implanted into pseudopregnant females. Chimeric male mice were bred to C57BL/6J females and germ line transmission of the targeted allele was examined in the agouti offspring by Southern blot and PCR analysis. Removal of the neo cassette, e.g. generation of mice with a floxed ERα allele ($ER\alpha^{flox/+}$), was performed by flp-assisted deletion *in vivo* by mating mice with the targeted allele with transgenic FLPe *deleter* mice (Artemis Pharmaceuticals GmbH). The name of the generated floxed ERα mouse line according to the ILAR nomenclature is B6.129X1-*Esr1*^{tm1Gust}. Mice with a deleted ERα allele were generated by crossing $ER\alpha^{flox/+}$ with transgenic Cre *deleter* mice as described previously [13]. After backcrossing into C57BL/6J mice, mice without the Cre transgene were used for further breeding. The name of the $ER\alpha^{-/-}$ mice according to the ILAR nomenclature is B6.129X1-*Esr1*$^{tm1.1Gust}$. All mice analyzed in this study were on a congenic C57BL/6J genetic background, e.g. backcrossed into C57BL/6J for 10 generations or more. Mice were maintained on a 14 h light, 10 h dark cycle and given continuous supply of food and water. The animal studies were approved by the Stockholm South ethical review board.

2.2. Genotyping of mice

DNA from tail or ear biopsies were used as templates in PCR reactions using primers P1 (ERα in1SP: 5′-GGAATGAGACTTGTCTATCTTCGT) and P2 (ERα 3′ASP: 5′-CCTGGCATTACCACTTCTCCT), which detects the wild type (WT) allele as a 748 bp product and the ERα deleted allele as 283 bp, whereas primers P1 and P3 (ERα ASP3: 5′-GACACATGCAGCAGAAGGTA) were used to detect the WT (size 205 bp) and floxed (size 310 bp) alleles. Presence of flp was assayed with primers Flpe-s: 5′-CACCTAAGGTCCTG GTTCGTCA and Flpe-as: 5′-CCCAGATGCTTTCACCCTCACT and Cre

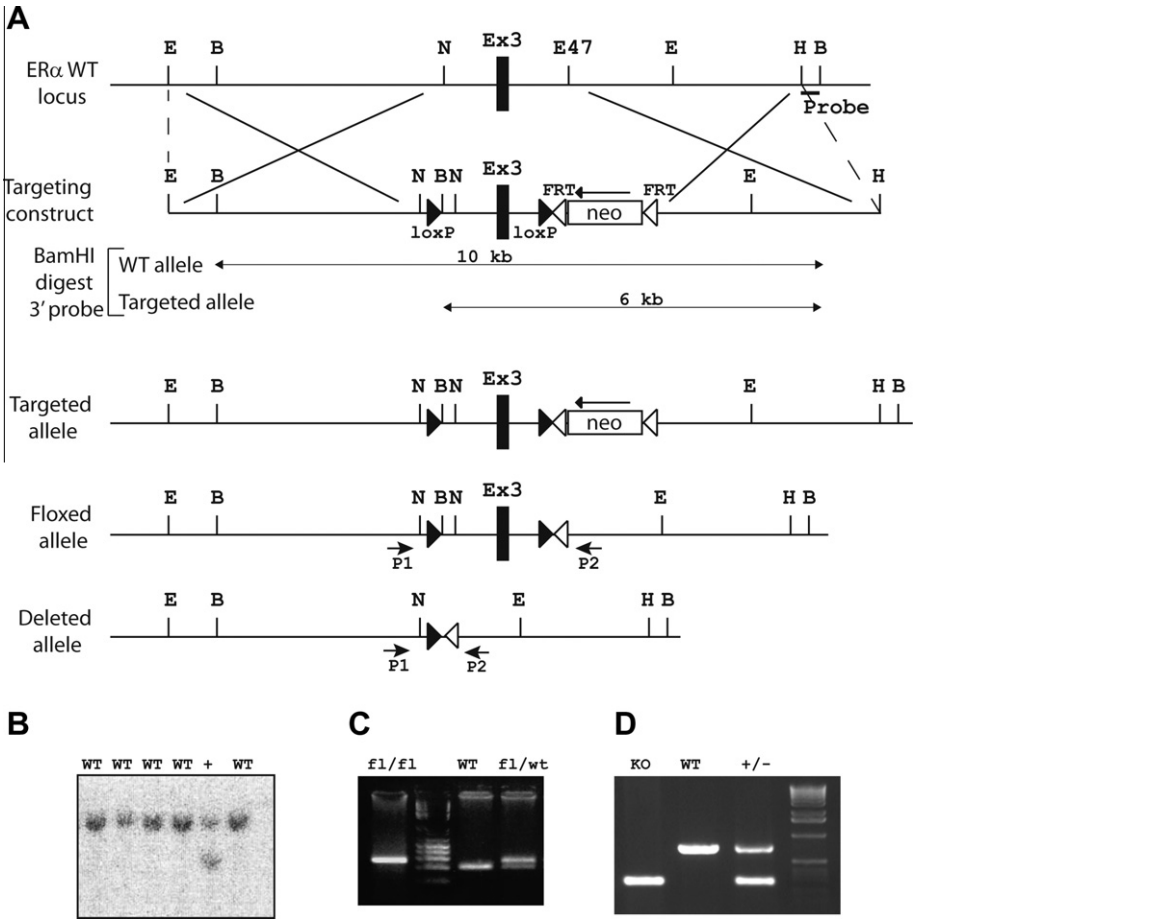

Fig. 1. Targeted disruption of the mouse ERα gene. (A) Structures of the WT ERα allele, targeting vector, targeted allele, floxed allele after flp-recombination, and deleted allele after Cre-recombination are shown with the EcoRV (E), BamHI (B), NheI (N), Eco47 (E) and HpaI (H) restriction sites and primers for PCR screening are indicated as arrows. (B) Homologous recombination in ES cells. Southern blot analysis of G418 resistant ES clones digested with BamHI is shown. The probe is indicated in (A). The 10 kb band for the WT allele and the 6 kb band for the targeted allele are indicated. (C) PCR genotyping of mouse DNA after flp recombination. The bottom band, using primers P1 and P2, represents the WT allele and the top band the ERα floxed allele. (D) PCR genotyping of mouse DNA after Cre recombination. The bottom band, using primers P1 and P2, represents the deleted allele and the top band the WT allele.

with Cre-sense: 5′-CCAATTTACTGACCGTACACC and Cre-antisense: 5′-GTTTCACTATCCAGGTTACGG.

2.3. RNA isolation, RT-PCR, DNA sequencing, and Western blot analysis

RNA was isolated using a kit (E.Z.N.A, Omega bio-tek). cDNA was synthesized using random hexamers and Superscript II (Invitrogen). PCR was done with high fidelity TAQ (Fermentas) with the following primers: exon2F: 5′-CCCTACTACCTGGAGAACGA with exon5R: 5′-TGCCCACTTCGTAACACTTG or exon9: R5′-CAGGGATTCTCAGAACCTTT. PCR products were cloned by TA cloning using pGEM-T easy (Promega) and sequenced at Macrogen Inc., South Korea. Tissue protein extracts were prepared with RIPA buffer (Sigma) and Western blot analysis was performed using standard protocols.

2.4. Fertility tests

Fertility tests of male ($n = 5$) and female ($n = 7$) mice were performed using continuous mating with WT partners for 6 months. Mating started when the mice were six weeks old and the number of pups and litters was recorded.

2.5. Antibodies

Rabbit polyclonal anti-ERα (H-184) and anti GAPDH (Santa Cruz Biotechnology Inc.) antibodies were used for Western blot analysis. Rabbit polyclonal anti-ERα (MC-20) (Santa Cruz Biotechnology Inc.) and the chicken polyclonal anti-ERβ503 antibody [14] were used for immunohistochemistry. Biotinylated anti-rabbit and anti-chicken antibodies were from Vector Laboratories (Burlingame, CA).

2.6. Immunohistochemical staining

The representative blocks of paraffin-embedded tissues were cut at 4 μm thickness, deparaffinized, and rehydrated. Antigens were retrieved by microwaving at 650 W in 10 mM citrate buffer (pH 7.0) for 15 min. The sections were incubated in 0.5% H_2O_2 in PBS for 30 min at room temperature to quench endogenous peroxidase, then incubated in 0.5% Triton X-100 in PBS for 15 min. To block nonspecific binding, sections were incubated in BlockAce (Dai-Nippon Pharmaceutical, Japan) for 40 min at room temperature. Sections were incubated with the following antibodies and dilutions: anti-ERα (1:500), anti-ERβ (1:250) in 10% BlockAce in PBS overnight at 4 °C. After washing, sections were incubated with biotinylated corresponding secondary antibodies (all in 1:200 dilutions) for 1 h at room temperature. The Vectastain ABC kit (Vector) was used for the avidin–biotin complex (ABC) method according to the manufacturer's instructions. Peroxidase activity was visualized with 3, 3′-diaminobenzidine (Dako). The sections were lightly counterstained with hematoxylin. Negative controls were incubated without primary antibody.

2.7. Whole-Mount Analysis of Mammary Glands

We examined changes in ductal morphology in mice at 10 weeks of age. Four mice (two WT and two ERα$^{-/-}$ mice) were compared. Excised abdominal mammary glands were spread on glass slides and fixed in a mixture of ethanol, chloroform, and glacial acetic acid (6:3:1 vol/vol) for 4 h at room temperature. The glands were then processed as follows: 70% ethanol for 15 min followed by rinsing in distilled water for 5 min and staining overnight at 4 °C in carmine alum solution (1 g of carmine red, 2.5 g of aluminum potassium sulfate in 500 ml of water). Stained glands were dehydrated in graded ethanol (70%, 95%, and 100% for 15 min at each step) and xylene for 1 h before mounting. Whole mounts were photographed using Leica dissecting microscope and video system.

2.8. Blood glucose analysis

Blood glucose concentrations were measured with the One-Touch Ultra glucometer (Accu-Chek Sensor, Roche Diagnostics).

2.9. Statistical analysis

All values are expressed as mean ± SD. Student́s *t*-test was used to identify significant differences between groups. The level of significance was set at $P < 0.05$.

3. Results

3.1. Generation of floxed ERα mice

To generate mice that allowed both conditional and global disruption of the ERα gene we used the Cre/loxP and flp/FRT recombination systems to target exon 3. Exon 3 of the ERα gene encodes the DBD of ERα and removal of this exon results in a frame shift of the coding region after splicing from exon 2 to exon 4. The

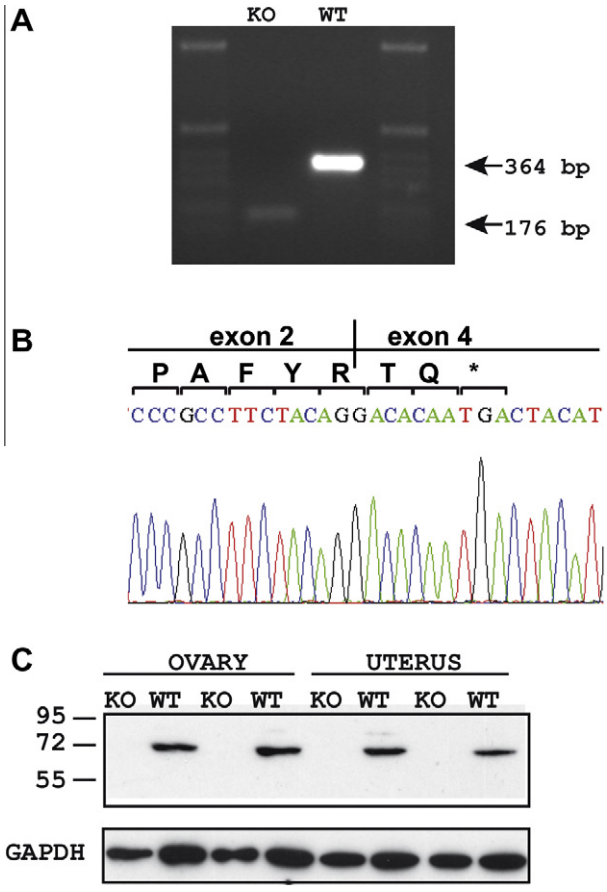

Fig. 2. Absence of ERα mRNA and protein in ERα knockout mice. (A) RT-PCR analysis of total RNA from WT and ERα$^{-/-}$ ovaries. Expression of ERα transcript is detected in WT ovary and a transcript lacking exon 3 in ERα$^{-/-}$ ovary. (B) DNA sequence determination of cDNA cloned from knockout mice. The sequence show that splicing between exons 2 and 4 occurs in knockout mice which generate a frame shift in the reading frame that results in an in frame stop codon indicated as *. (C) Western blot analysis of tissue extracts from ovary and uterus using ERα polyclonal antibodies. ERα protein, indicated by an arrow, is detected in WT but not in null tissues while GAPDH is detected in all samples.

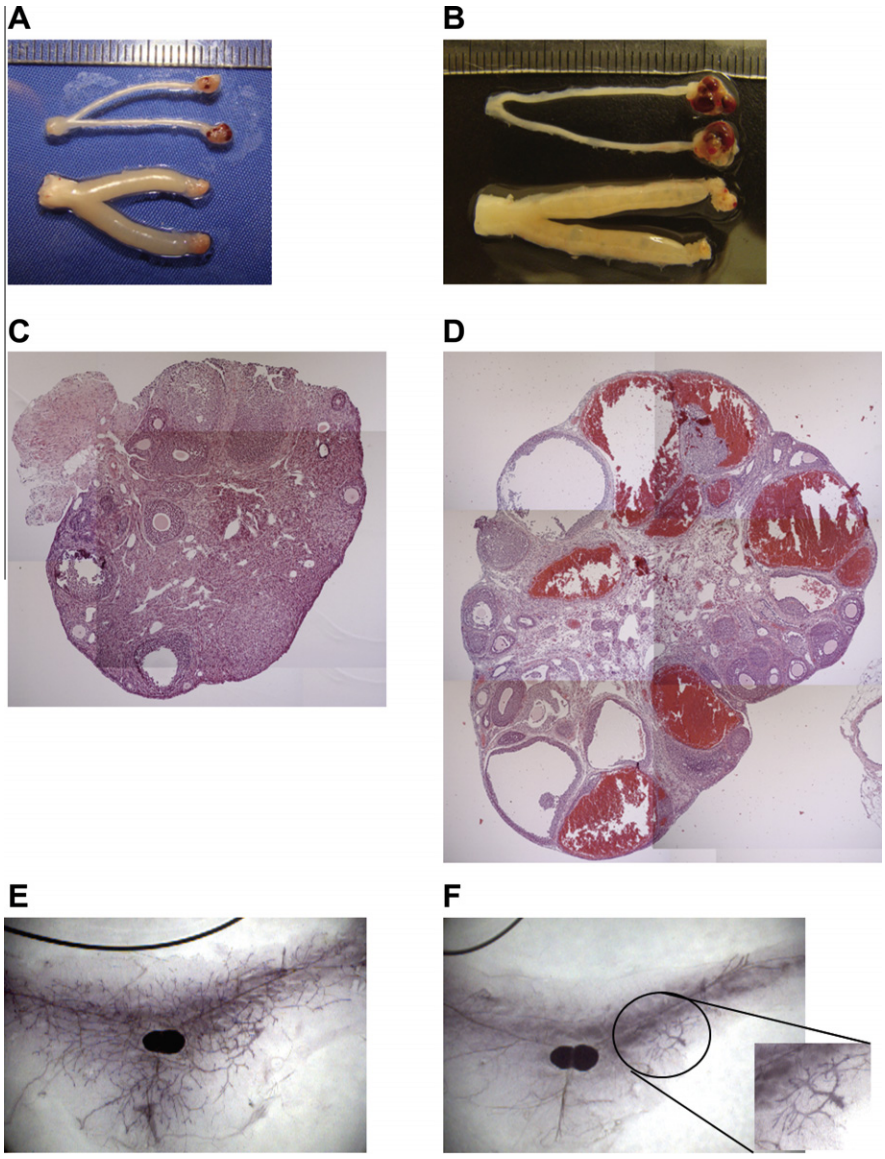

Fig. 3. Developmental defects in ERα$^{-/-}$ uterus, ovaries and mammary glands. Photos of ovaries and uterus from WT and ERα$^{-/-}$ mice at 10 weeks (A) and 1 year (B) of age. ERα$^{-/-}$ mice have rudimentary uterus and hemorrhagic polycystic ovaries. H&E staining was used to analyze the morphology of WT (C) and ERα$^{-/-}$ (D) ovaries from 12 weeks old mice. Ovaries from WT mice have many follicles of various stages and corpora lutea. Ovaries from ERα$^{-/-}$ mice have early stages of follicles but no matured follicles and no corpora lutea. Instead of matured follicles, there are many hemorrhagic cystic follicles. Whole mount staining of mammary glands from 10 weeks old mice. Ductal elongation was observed till end of mammary fat pads in WT (E) whereas elongation did not reach till lymph node in ERα$^{-/-}$ mice (F).

putative translated truncated protein would lack both the DBD and the LBD and only express the N-terminal 155 amino acids of ERα. The targeting vector was designed to introduce a loxP site in intron 2 and an FRT flanked neomycin cassette with a loxP site in intron 3 (Fig. 1A). ES cells were electroporated with targeting vectors and selected with G418. Resistant clones were isolated and analyzed for homologous recombination by Southern blot analysis using a 3′ external probe (Fig. 1B). Two targeted ES cells lines were injected into C57BL/6 blastocysts to produce chimeric mice. Both clones contributed to the germ line e.g. produced agouti offspring. To generate floxed ERα mice, ERα$^{flox/+}$, the neomycin cassette was then removed *in vivo* by using flp/FRT recombination by crossing neo positive offspring from chimeric breeding with transgenic flp *deleter* mice. The correct recombination was verified by PCR (Fig. 1C) and sequence determination of the PCR products (data not shown). The progeny of these mice were backcrossed into C57BL/6J mice for 10 generations to generate fully backcrossed heterozygous floxed ERα mice.

3.2. Generation of ERα knockout mice

To generate mice with a deleted ERα allele, ERα$^{flox/+}$ mice were crossed with transgenic cre *deleter* mice. Correct recombination and removal of exon 3 in the offspring was determined by PCR (Fig. 1D) and sequence determination of the PCR products (data not shown). After further breeding with WT mice, mice that lacked both exon 3 and the Cre transgene were selected for maintaining the colony. ERα knockout mice, ERα$^{-/-}$ mice, were generated by heterozygous breeding of ERα$^{+/-}$ mice.

3.3. Verification of the null mutant in ERα$^{-/-}$ mice

To verify that exon 3 of the ERα gene was not expressed in ERα$^{-/-}$ mice we used RT-PCR analysis on RNA extracted from ovaries using primers in exons 2 and 5. ERα mRNA was present in ovary from WT mice but ovaries from ERα$^{-/-}$ mice expressed a shorter transcript (Fig. 2A). Sequence determination of the

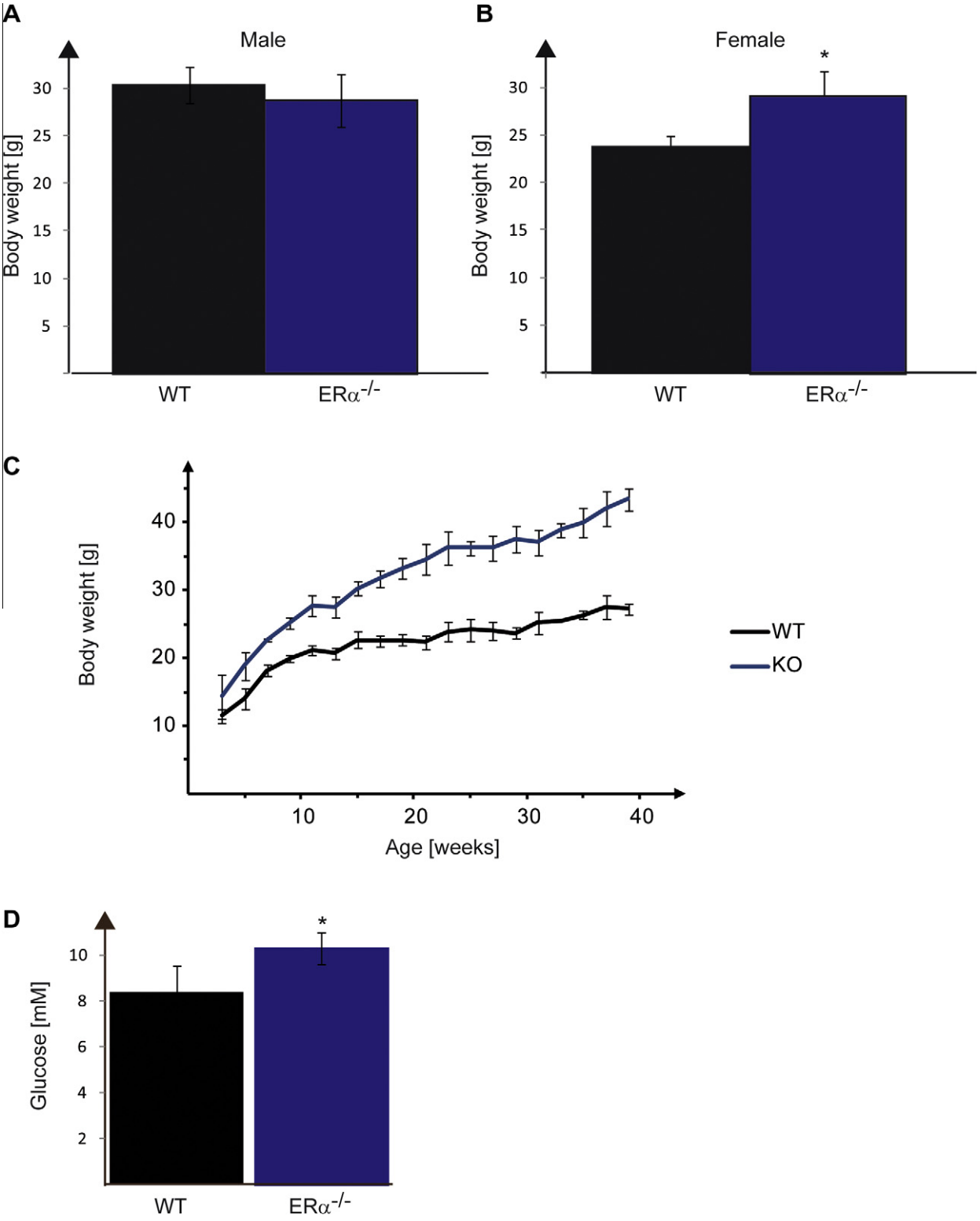

Fig. 4. Body weights and blood glucose concentration of female ERα$^{-/-}$ mice and WT controls. (A) Body weights of male and female WT and ERα$^{-/-}$ mice at 4 months of age. Values are presented as mean ± SD (n = 8). (B) Weight determination of female mice up to nine months of age. Values are presented as mean ± SD (n = 3). (C) Blood glucose levels in 3 months old female mice (n = 7) presented as mean ± SD. *P < 0.01.

ERα$^{-/-}$ transcript showed that this transcript lacked exon 3 and that splicing occurred between exons 2 and 4 which gives rise to an in frame stop codon at the beginning of exon 4 (Fig. 2B). The predicted putative translated protein would express the first 155 amino acids from ERα and two extra amino acids from the frame shift in exon 4 and would neither have a DBD nor an LBD. We also used a primer overlapping the stop codon in exon 9 together with the exon 2 primer and detected only single bands suggesting that no alternative splicing occurred. To verify that no ERα protein was expressed we used Western blot analysis with tissue lysates

from ovary and uterus that are tissues known to express high levels of ERα. In extracts from WT tissue a 66 kDa single band was detected but this band was absent in extracts from ERα$^{-/-}$ mice (Fig 2C). The lack of ERα protein was further confirmed by immunohistological analysis which detected ERα staining in uterus (endometrial and stromal cells), ovary (theca cells) and mammary glands (luminal cells) from WT mice but not in tissues from ERα$^{-/-}$ mice (Fig. S1).

3.4. Sterility and defects in reproductive system in ERα$^{-/-}$ mice

To test fertility in ERα$^{-/-}$ mice, five male and seven female ERα$^{-/-}$ mice were continuously mated with fertile males/females for 6 months. No litters or pups were recorded (data not shown) which is in agreement with studies of other ERα knockout mouse lines. Morphological analysis of reproductive tract from ERα$^{-/-}$ mice showed that 10 weeks old ERα$^{-/-}$ mice have rudimentary uterus and hemorrhagic polycystic ovaries (Fig. 3A). In 1 year old ERα$^{-/-}$ mice the uterus is severely hypoplastic and ovaries developed more serious hemorrhagic cysts (Fig. 3B). The section of WT ovary showed various stages of follicles and corpora lutea (Fig. 3C). Ovary from ERα$^{-/-}$ mice showed early stage of follicles but no matured follicles and no corpora lutea. Instead of matured follicles, there were many hemorrhagic cystic follicles (Fig. 3D). In the mammary glands ductal elongation normally begins when mice are 3 weeks old and is completed by 7 weeks of age. Ductal elongation was observed till edge of mammary fat pads in WT mice (Fig. 3E) whereas elongation did not reach till lymph node in ERα$^{-/-}$ mice (Fig. 3F).

3.5. Increased body weight in female ERα KO mice

Female ERα$^{-/-}$ mice develop obesity and at 4 months of age there was a significant increase in body weight of ERα$^{-/-}$ female mice compared to WT controls, but there were no significant difference between male WT and ERα$^{-/-}$ mice at this age (Fig. 4A&B). The difference between WT and ERα$^{-/-}$ female mice increased with age and at 9 months of age the KO mice were more than 50% heavier (Fig. 4C). We also show that the basal blood glucose levels are more than 20% higher in female ERα$^{-/-}$ mice compared to WT controls (Fig. 4D).

4. Discussion

In this study we have developed a mouse line with a conditional allele of ERα and used these mice to make a new ERα knockout mouse line by crossing them with transgenic Cre mice. These knockout mice lack ERα exon 3 which codes for the first zinc finger in the DBD and we verified this deletion by PCR and sequence determination of genomic DNA from ERα$^{-/-}$ mice. Furthermore, using RT-PCR we showed that, as a result of this deletion, mRNA splicing occurred between exons 2 and 4 producing a truncated transcript with a frame shift that introduces a stop codon at the beginning of exon 4, before the LBD (Fig. 2B). No ERα protein could be detected in tissues from ERα$^{-/-}$ mice using Western blot analysis or immunohistochemistry indicating that the knockout is complete which is in contrast to the first generated ERα KO mouse line that that was reported to have residual ERα activity due to splicing using cryptic splice sites [7]. The lack of ERα activity in our ERα$^{-/-}$ model was further confirmed by analyzing ERα$^{-/-}$ mice which displayed many of the expected phenotypes.

Our fertility tests showed that both male and female ERα$^{-/-}$ mice are infertile. Female infertility in ERα knockout mice has been found in all described ERα knockout mouse models [6,8,10,11,15] and male infertility has been shown in ERα knockout mice targeting exon 3 [8,15–17]. The infertility in female mice is likely to be caused by abnormalities in reproductive organs and we show that both ovaries and uterus have severe defects. The uterus is hypoplastic in ERα$^{-/-}$ mice and ovaries contain hemorrhagic cysts and lack mature follicles and corpora lutea. These defects are likely to be a result of a combination of direct effects of ERα deficiency in ovary and other organs in the hypothalamo–pituitary–ovarian axis. ERα is mainly expressed in theca cells in the ovary and theca cell specific deletion of ERα affects regulation of female reproduction but do not cause infertility [18]. However, deletion of ERα in neurons [12,19] or pituitary [20] have been shown to result in ovarian defects and infertility. We also show defective development of mammary glands in female ERα$^{-/-}$ mice. Feng et al [9] showed a direct role of ERα in mammary gland development using tissue specific knockouts.

The female ERα$^{-/-}$ mice develop obesity which is in agreement with the known role of estrogens and ERα as regulators of metabolism, reviewed in [21], and also in agreement with other studies [10,11,22,23]. We show that female mice have increased basal glucose levels and these are likely to contribute to the obesity. To date it is not known exactly which organs that cause the metabolic defects resulting in increased body weight in female ERα$^{-/-}$ mice, but recent studies using conditional knockouts point to specific roles for ERα in both CNS and myeloid cells that affect both glucose homeostasis and control of body weight [19,24].

In summary, we have generated a floxed ERα mouse model and used this to make a mouse line with global knockout of the ERα gene. The global ERα$^{-/-}$ mice show all expected phenotypes such as infertility and severe defects in ovaries, uterus and mammary glands as well as obesity. Collectively these results indicate that our new ERα$^{-/-}$ model is appropriate for studies of estrogen signaling and that the floxed ERα mice will be valuable tools for future studies using tissue specific knockouts.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2012.07.016.

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