ORIGINAL ARTICLE

Reduced prostate branching morphogenesis in stromal fibroblast, but not in epithelial, estrogen receptor α knockout mice

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Early studies suggested that estrogen receptor alpha (ERα) is involved in estrogen-mediated imprinting effects in prostate development. We recently reported a more complete ERα knockout (KO) mouse model *via* mating β-actin *Cre* transgenic mice with floxed ERα mice. These ACTB-ERαKO male mice showed defects in prostatic branching morphogenesis, which demonstrates that ERα is necessary to maintain proliferative events in the prostate. However, within which prostate cell type ERα exerts those important functions remains to be elucidated. To address this, we have bred floxed ERα mice with either fibroblast-specific protein (FSP)-Cre or probasin-Cre transgenic mice to generate a mouse model that has deleted *ERα* gene in either stromal fibroblast (FSP-ERαKO) or epithelial (pes-ERαKO) prostate cells. We found that circulating testosterone and fertility were not altered in FSP-ERαKO and pes-ERαKO male mice. Prostates of FSP-ERαKO mice have less branching morphogenesis compared to that of wild-type littermates. Further analyses indicated that loss of stromal ERα leads to increased stromal apoptosis, reduced expression of *insulin-like growth factor-1* (*IGF-1*) and *FGF10*, and increased expression of *BMP4*. Collectively, we have established the first *in vivo* prostate stromal and epithelial selective ERαKO mouse models and the results from these mice indicated that stromal fibroblast ERα plays important roles in prostatic branching morphogenesis *via* a paracrine fashion. Selective deletion of the *ERα* gene in mouse prostate epithelial cells by probasin-Cre does not affect the regular prostate development and homeostasis.
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INTRODUCTION

Prostate growth and development are primarily controlled by androgens. During prostatic development, in the presence of androgens/androgen receptor (AR),[1] the urogenital mesenchyme (UGM) directs prostatic epithelial budding and branching morphogenesis through interactions between the urogenital sinus epithelium and UGM.[2] Conversely, the developing prostatic epithelium induces fibroblasts to undergo smooth muscle differentiation.[3] In addition to androgens, the prostate can also respond to the stimulation of estrogens.[4–6] Lower doses of estrogens have been reported to increase prostate weight and prostatic budding.[5,6] In contrast, higher doses of estrogens permanently suppress the neonatal rat or mouse prostate growth and prostatic branching morphogenesis by reducing testosterone (T) production, suppressing AR expression, and inducing prostatic epithelial regression.[7–10] Estrogen's actions can be mediated by estrogen receptor alpha (ERα) and ERβ,[11,12] encoded by two distinct genes. Studies conducted with conventional neomycin (Neo) gene knockin and ER knockout (KO) mice suggested that ERα, but not ERβ, acts as a dominant receptor of estrogen signaling in males for the development of prostate and for maintenance of normal reproductive functions.[13–16] However, it remains unclear how ERα functions in different types of prostate cells to affect prostate development. Stromal–epithelial recombination experiments were used to determine ERα functions in stromal *vs.* epithelial cells. But, there are two major concerns with results obtained from those tissue recombinant experiments: (i) the stromal and epithelial cells used for the recombination experiment may not represent the prostate cell origins; and (ii) the recombinant is implanted under the renal capsule of immunodeficient mouse for a short period of time. Therefore, those experiments may not completely recapitulate the *in vivo* ERα roles in prostate developments. To resolve those potential pitfalls, floxed ERα mice were produced using a self-excising ACN (tACE-Cre/Neo) cassette.[17] We successfully produced the total ERαKO mice by mating floxed ERα mice with β-actin-Cre mice (ACTB-ERαKO), and proved that deletion of the floxed exon 3 allele can disrupt the reading frame of the ERα transcript resulting in undetectable ERα protein.[17] Studies of ACTB-ERαKO male mice demonstrated that ERα is required for maintaining male fertility, prostatic branching morphogenesis and the homeostasis of the prostate.[18] The mature prostate consists of various cell types including luminal epithelial, basal epithelial, neuroendocrine, smooth muscle, fibroblast, vascular endothelial cells, etc. In

the rodent prostate, ERα is detectable in both epithelial and stromal cells.[16,19–21] To date, several studies focused on ERα roles in high-dose estrogen-mediated squamous metaplasia (SQM) and carcinogenesis.[20,22,23] However, no *in vivo* model has effectively evaluated the roles of epithelial *vs.* stromal fibroblast ERα during normal prostate development. The establishment of the floxed ERα mouse model allows us to selectively delete the *ERα* gene in a tissue- or temporal-specific manner by breeding with transgenic mice harboring promoter specific *Cre*.[18] We now report the generation of the first mouse model that has floxed *ERα* gene selectively knocked out in stromal fibroblast cells *via* breeding with fibroblast-specific protein 1-*Cre* (FSP-*Cre*) or

in epithelial cells *via* breeding with prostate epithelial-specific proba-sin-*Cre* (pes-*Cre*) to dissect the stromal *vs.* epithelial ERα function. Our *in vivo* tissue-selective ERαKO mouse models demonstrate that stromal fibroblast ERα plays an important role in prostatic branching morphogenesis *via* a paracrine-regulatory fashion.

MATERIALS AND METHODS

Generation of tissue selective ERαKO Mice

To generate FSP-*Cre*/ERα^{fl/fl} and pes-*Cre*/ERα^{fl/fl} mice (FSP-ERαKO and pes-ERαKO), we mated floxed ERα homozygous female mice[17] with FSP-*Cre* and probasin-*Cre* transgenic male mice, respectively.
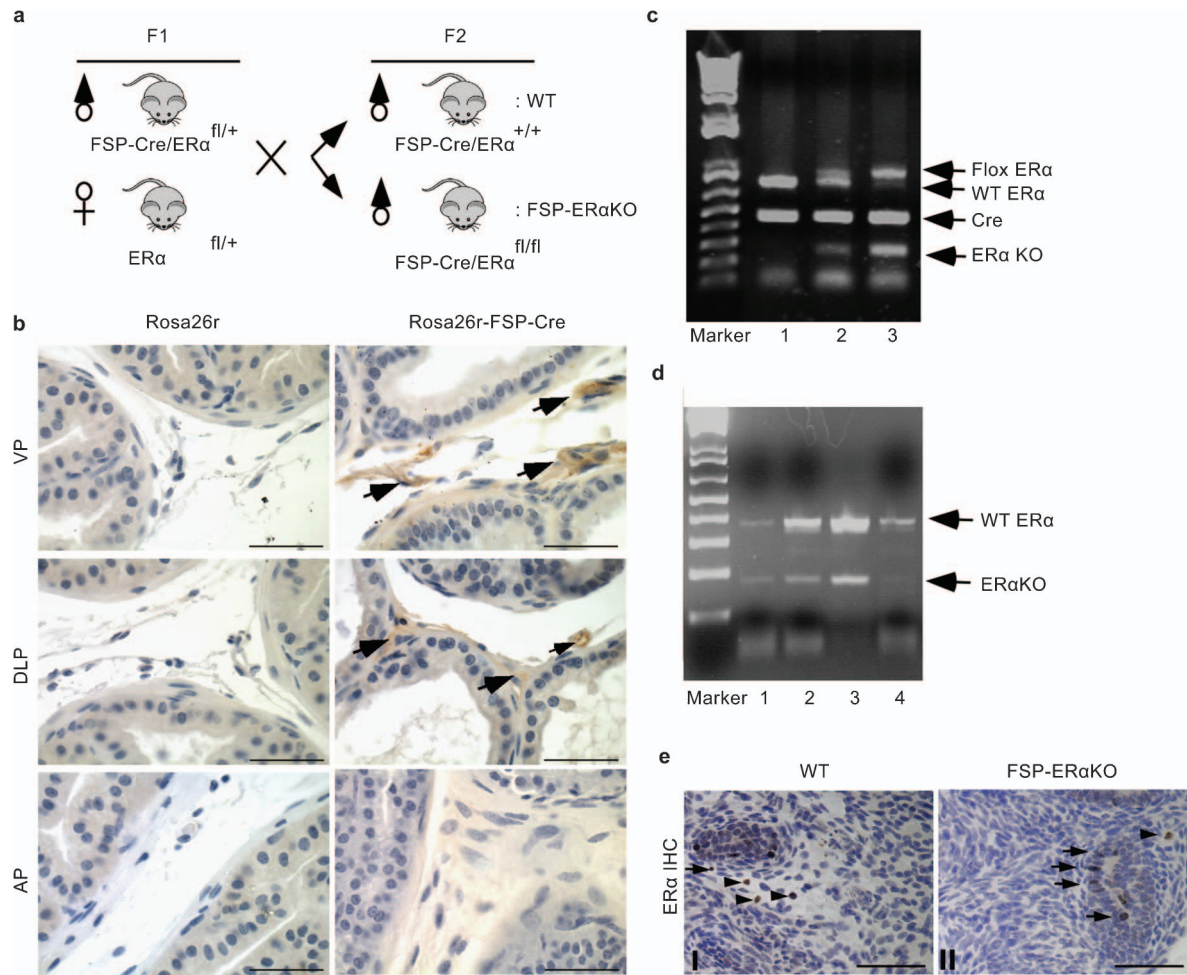

Figure 1 Generation and genotyping of FSP-ERαKO *via* Cre-loxP strategy. (**a**) Breeding strategy to generate the tissue-specific ERαKO mice. Floxed ERα homozygous female mice were mated with FSP-Cre transgenic male mice to obtain the FSP-Cre/ERα^{fl/+} male mice. FSP-Cre/ ERα^{fl/+} male mice were mated with floxed ERα heterozygote female mice (F1) to obtain the FSP-Cre/ERα^{+/+} (WT) and FSP-Cre/ERα^{fl/fl} (KO) mice (F2). (**b**) FSP-Cre-mediated specific Cre deletion of the floxed stop codon and the expression of β-galactosidase in the prostate stromal fibroblasts of the Rosa26r-FSP-Cre mice. β-galactosidase expression mediated by FSP-Cre excision was observed in stromal compartment of VP and DLP (arrows), but not in the stromal compartment of AP. Immunohistochemical staining of β-galactosidase was detected using β-gal antibody (Iowa University Hybridoma Bank, Iowa city, IA, USA). Scale bar is equal to 25μm. (**c**) Genotypes of tail snips of FSP-Cre/ERα^{fl/fl} mice. Lane 1: FSP-Cre/ERα^{+/+} mice; lane 2: FSP-Cre/ERα^{fl/+} mice; lane 3: FSP-Cre/ERα^{fl/fl} mice. The *FSP1* gene promoter is selectively expressed in the fibroblast cells, explaining the presence of the KO allele in the tail snips of FSP-Cre/ERα^{fl/+} and FSP-Cre/ERα^{fl/fl} mice (lanes 2 and 3), in which intervening DNA of floxed ERα allele was partially deleted by FSP-Cre recombinase. The size of deleted ERα allele was reduced compared to WT and floxed ERα. The size of floxed ERα, WT ERα, ERα KO allele, and Cre are 881bp, 741bp, 223bp, and 411 bp, respectively. (**d**) Detection of ERα transcripts in the various adult male reproductive organs was performed by RT-PCR using primers that span exons I and III of the *ERα* gene in the FSP-ERαKO mice. Lane 1: prostate; lane 2: epididymides; lane 3: efferent ducts; lane 4: testes. In FSP-ERαKO mice; both WT ERα band and the truncated transcript band (deletion of exon 3) can be detected in different male reproductive organs due to floxed *ERα* gene KO in fibroblasts. (**e**) The expression of ERα in the 1-week-old FSP-ERαKO and WT VPs was determined by IHC. Arrows show positive staining of ERα in the epithelial cells. Arrowheads show positive staining of ERα in the stromal cells. Scale bar is 100 μm; AP, anterior prostate; DLP, dorso-lateral prostate; ER, estrogen receptor; FSP, fibroblast-specific protein; IHC, immunohistochemistry; KO, knockout; RT-PCR, real-time PCR; VP, ventral prostate; WT, wild type.

FSP-*Cre* mice were received from Drs H. L. Moses and N. A. Bhowmick (Vanderbilt-Ingram Cancer Center, Nashville, TN, USA) and the probasin-*Cre* mice were obtained from National Cancer Institute (Bethesda, MD, USA). ERα$^{fl/fl}$ mice were created through a collaboration with Dr S. Radovick and Dr A. Wolf (Johns Hopkins University, Baltimore, MD, USA).[17,18] After two generations of mating, FSP-ERαKO (**Figure 1a**) and pes-ERαKO (**Figure 4a**) mice were developed. Genomic DNA was isolated from tail biopsies and used as a template for PCR genotyping. Genotyping and primers were listed in our previously publications.[17,18] The Rosa26r reporter mice have the *lacZ* gene inserted into the ubiquitously expressed Rosa locus. This locus is preceded by a transcriptional stop cassette flanked by two loxP sites. When *Cre*-recombinase is expressed, β-Gal activity can be observed in target organs.[23,24] We bred Rosa26r-FSP-*Cre* compound mice to detect the *Cre* activity and expression pattern of FSP-*Cre* mice. The ACTB-ERαKO, a whole-body ERαKO, mice were generated by breeding flox ERα mice with β-actin-*Cre* mice as previously described.[17,18] 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.

Tissue dissection and prostatic ductal tip count

Mice were first anesthetized to collect serum for hormone analysis and then killed by CO_2 asphyxiation. After centrifugation, the serum was frozen at −70 °C until assayed. The genital tracts of the animals were exposed by a lower abdominal incision. The individual organs were then microdissected, weighed, photographed and frozen for further analysis, or fixed in formalin for subsequent histological examination. For the ductal tip count experiment, microdissected ventral prostate (VP), dorso-lateral prostate (DLP) and anterior prostate (AP) were incubated in Hank's buffer containing 1% collagenase at 37 °C for 1 h to digest and remove stromal compartment. The number of ductal tips was then counted under a dissecting microscope.

Hormone radioimmunoassay

All male mice were housed individually for 1 week before collection of serum for hormone analysis. Serum T was determined using a T double antibody radioimmunoassay kit (Diagnostic System Laboratories, Inc., Webster, TX, USA). The lowest detectable T concentration is 2.0 pg ml^{-1}. Animals used in the hormone studies were approximately 3 months of age.

Sperm counting

The mouse epididymides were chopped into small pieces in 1.5 ml RPMI media, incubated for 40 min at 37 °C to release the sperm, and then diluted for counting the total sperm numbers per epididymis.

Immunohistochemistry (IHC), immunofluorescent staining and terminal transferase-mediated DNA end labeling (TUNEL) assay

IHC and immunofluorescent staining were carried out as described previously.[17,18] Sections were incubated with the following antibodies and dilutions: anti-β-gal antibody (40-1A, 1 : 400), anti-ERα (MC-20, 1 : 400), anti-AR (N-20, 1 : 400), anti-Ki67 (NCL-Ki67p, 1 : 1000), anti-vimentin (LN-6, 1 : 200), anti-SM α-actin (1A4, 1 : 400), anti-desmin (DE-U-10, 1 : 300), anti-heavy chain myosin (G-4, 1 : 500) and anti-pan-cytokeratin (H-240, 1 : 200) in phosphate buffered saline containing 3% bovine serum albumin overnight at 4 °C. For IHC, the tissue slides were incubated with 1 : 300 diluted biotinylated secondary antibody (Vector Laboratories, Burlingame, CA, USA) and ABC solution (Vector Laboratories), then stained using AEC (DAKO,

Carpenteria, CA, USA). Mayer's hematoxylin was used for a nuclear counterstain. Negative controls were incubated without primary antibody. For immunofluorescent staining, the tissue slides were incubated with 1 : 100 Texas red-conjugated goat anti-mouse IgG or fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (ICN, Costa Mesa, CA, USA). Stained slides were mounted with mounting media containing 4′-6-Diamidino-2-phenylindole and visualized with a fluorescent microscope. The apoptosis in paraffin embedded tissue was detected by the TUNEL assay using *In situ* Cell Death Detection Kit (Roche, Indianapolis, IN, USA) following the manufacturer's instructions. 4′-6-Diamidino-2-phenylindole-stained cells were counted under a fluorescent microscope from at least six randomly selected fields. Cells positive for TUNEL staining were counted in the same field. At least six fields from each lobe of prostates were analyzed. The apoptotic index was determined by dividing the number of TUNEL positive cells by the total numbers of cells in all six fields.

RNA extraction and RT-PCR

Total RNA was extracted and purified using Trizol (Invitrogen, Carlsbad, CA, USA) and RT-PCR has been described previously.[18,25] Briefly, 3 μg total RNA from wild-type (WT) or tissue-specific ERαKO prostates was subjected to reverse transcription using Superscript III (Invitrogen, Carlsbad, CA, USA). The RT-PCR was performed with first-strand cDNA, specific gene primers and SYBR Green PCR Master Mix (Biorad, Hercules, CA, USA). The Q-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 RT-PCR detection system (Biorad). Primer sequences for mouse genes have been described previously.[18] The primer sequences for rat genes: *FGF7*, sense, 5′-GCTTCCACCTCGTCTGTCTTG-3′; antisense, 5′-CCCTTTCACTT-TGCCTCGTTTG-3′. *FGF10*, sense 5′-CTGTTGCTGCTTCTTGTTG-3′; antisense, 5′-TTCCACTGATGTTATCTCTAGG-3′. *IGF-1*, sense, 5′-CTGCTGCGTGGTGGTCTG-3′; antisense, 5′-CATTGTATGG-CTATCTGTCTTGGC-3′. *BMP4*: sense, 5′-TGATACCTGAGAC-CGGGAAG-3′; antisense, 5′-AGCCGGTAAAGATCCCTCAT-3′. *BMP7*, sense, 5′-TCCGGTTTGATCTTTCCAAG-3′; antisense, 5′-TGGTGGCTGTGATGTCAAAT-3′. *β-actin*: sense, 5′-TATCGG-CAATGAGCGGTTCC-3′; antisense, 5′-TATCGGCAATGAGCGG-TTCC-3′. Each sample was run in triplicate for each Q-PCR. Data were analyzed using iCycler iQ software (Biorad).

Statistical analyses

Values were expressed as mean±standard deviation (s.d.). We used the *t*-test to compare values among the two groups. Calculated *P* values were two-sided, and $P < 0.05$ was considered statistically significant.

RESULTS

Generation of FSP-ERαKO Mice

To address the role of stromal fibroblast ERα in normal prostate development, we generated mice with conditional deletion of the *ERα* gene in stromal fibroblast cells by mating floxed ERα female mice with male mice expressing *Cre* recombinase driven by the FSP1 promoter (**Figure 1a**). FSP1 gene expression is first detected by *in situ* hybridization at embryonic day 8.5 as a postgastrulation event, and is associated with cells of mesenchymal origin or of fibroblastic phenotype.[26–28] Selective *Cre*-mediated recombination exerted by the FSP-*Cre* transgene has been observed in stromal fibroblast cells throughout the mouse, including the prostate, forestomach and skin.[29] The Rosa26r reporter mice have the *lacZ* gene inserted into the ubiquitously expressed Rosa locus, which is preceded by a transcriptional

stop cassette flanked by two loxP sites. When *Cre*-recombinase is expressed, this stop cassette is deleted and β-galactosidase activity can be observed in target organs.[23,24] We examined FSP-*Cre*-mediated loxP site recombination within the mouse prostates in Rosa26rFSP-*Cre* mice. **Figure 1b** showed that FSP-*Cre* specifically expressed in the prostatic stromal compartment with strong expression in the VP and DLP (arrows), marginal expression in the AP, and was not detectable in the prostatic epithelial cells.

To verify *ERα* gene deletion in the FSP-ERαKO mice, pups were genotyped for floxed ERα, WT ERα, ERαKO allele and the FSP-*Cre* transgene (**Figure 1c**). Deletion of ERα exon III was confirmed by detection of truncated mRNA transcripts using a pair of PCR primers located on exons II and IV of the *ERα* gene. Our data showed truncated ERα transcripts within the prostate, epididymis, efferent ducts and testis of the FSP-ERαKO mice (**Figure 1d**).

ERα deletion in FSP-ERαKO prostates was further validated by IHC in the prostates of 1-month-old mice. We found that ERα expression was positive in epithelial cells in VPs of WT and FSP-ERαKO mice (**Figure 1e**, I, arrows). However, ERα expression was dramatically reduced in the stromal compartments of FSP-ERαKO VPs (**Figure 1e**, II *vs.* I, arrowhead) as compared to that of WT littermates.

Normal genital tract development and fertility in adult FSP-ERαKO males

Comparing 3-month-old FSP-ERαKO males to WT littermates, histological analyses demonstrated that there were no phenotype alterations in the male reproductive system including testes, epididymides (**Figure 2a**), or efferent ducts and seminal vesicles (data not shown). FSP-ERαKO testes consisted of compact seminiferous tubules. The atrophic and degenerating seminiferous tubules present in the ACTB-ERαKO were not observed in the FSP-ERαKO mice.[18] The FSP-ERαKO epididymis also appeared normal as compared to those of WT mice (**Figure 2a**). There was no statistical difference in the epididymal sperm number count between FSP-ERαKO and WT mice (**Figure 2b**). Furthermore, in contrast to the infertility of ACTB-ERαKO mice, when mating WT and FSP-ERαKO males with fertile B6 females, we found that there were no statistical differences in litter sizes among those matings (**Figure 2c**). Moreover, the serum T level in FSP-ERαKO males is similar to the levels in WT males at 3 months of age (**Figure 2d**). Together, our data indicate that the serum T levels, the development of testes and epididymides, and male fertility remain normal in FSP-ERαKO males.

Reduced prostate branching morphogenesis and weight in FSP-ERαKO mice

Our previous report[18] of the total ERαKO (ACTB-ERαKO) mice showed that ERα is involved in prostatic branching morphogenesis; however, it is unclear as to whether epithelial or stromal ERα plays a dominant role in prostatic branching morphogenesis. Results of comparing microdissected prostates from 3-month-old mice, demonstrated the reduced number of ductal tips derived from prostatic branching of adult ACTB-ERαKO prostates (**Figure 3a**, ACTB-ERαKO *vs.* WT, **$P < 0.001$ in both VP and DLP). Compared to WT mice, FSP-ERαKO mouse prostates had a significantly reduced branching morphogenesis (**Figure 3a**, FSP-ERαKO *vs.* WT, **$P < 0.01$ in VP; *$P < 0.001$ in DLP). It has been known that APs, also called coagulating glands, are less similar to human prostate structure and development. Indeed, we did not observe a difference in branching morphogenesis in
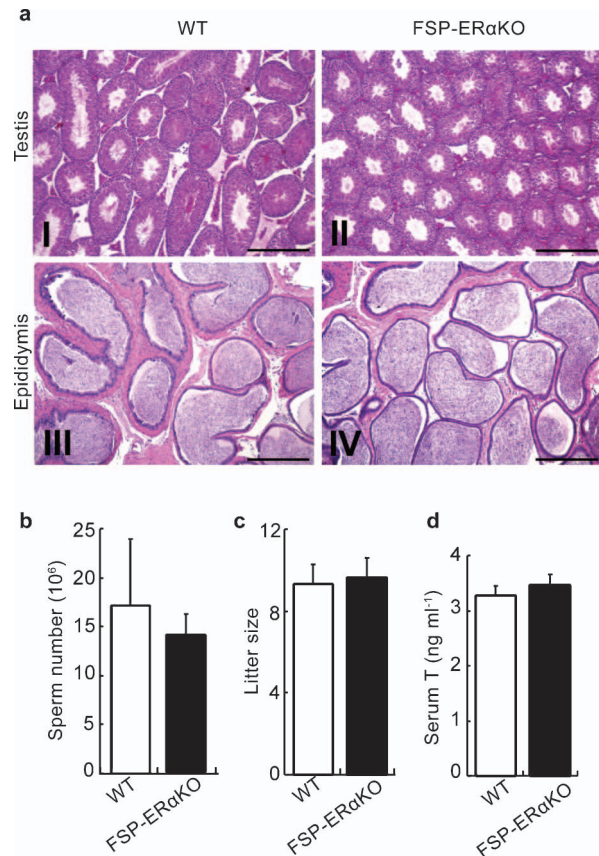
Figure 2 Histological analysis of reproductive organs and fertility test in WT (FSP-Cre/ERα$^{+/+}$) and FSP-ERαKO (FSP-Cre/ERα$^{fl/fl}$) adult males. (**a**) Histological analyses of testes and epididymides from adult WT and FSP-ERαKO males at the age of 3-month. FSP-ERαKO testes appeared normal and consisted of compact seminiferous tubules (II *vs.* I). Scale bar is 400 μm. The cauda epididymides from FSP-ERαKO males contained sperm comparable to WT mice (IV *vs.* III). Scale bar is 100 μm. (**b**) The sperm counts of adult FSP-ERαKO VP and WT littermates. Results are presented as mean±s.d. (*n*=5). (**c**) To assess fertility, 3-month-old WT or FSP-ERαKO males were mated with WT females. Pups per litter from each mating were compared and found to be similar. Results are presented as mean±s.d. (*n*=5). (**d**) The serum T levels in the adult FSP-ERαKO males. The serum samples were from 12- to 14-week-old males. Results are presented as mean±s.d. (*n*=4). ER, estrogen receptor; FSP, fibroblast-specific protein; IHC, immunohistochemistry; KO, knockout; T, testosterone; VP, ventral prostate; WT, wild type.

the APs of FSP-ERαKO and WT mice, which is consistent with results in the ACTB-ERαKO mice.[18] Consistently, we observed the reduced prostate weights in FSP-ERαKO mice (**Figure 3b**). Together, our findings from FSP-ERαKO provided direct evidence that prostate fibroblast ERα plays important roles in prostatic branching morphogenesis.

Increased stromal apoptosis in the FSP-ERαKO prostates

The mouse prostate undergoes extensive branching morphogenesis and 85% of the adult number of ductal tips and branch points are formed during the first 15 days after birth,[30] suggesting that prostate cell proliferation rate is high during the neonatal stages. As we observed reduced branching morphogenesis and prostate weights in the FSP-ERαKO mice (**Figure 3a** and **b**), we were interested in comparing the proliferation activity of the VP between FSP-ERαKO and WT mice at 1-week-old (**Figure 3c**). IHC results of Ki67 expression suggested that there was no difference in either epithelial proliferation (**Figure 3d**, arrows) or
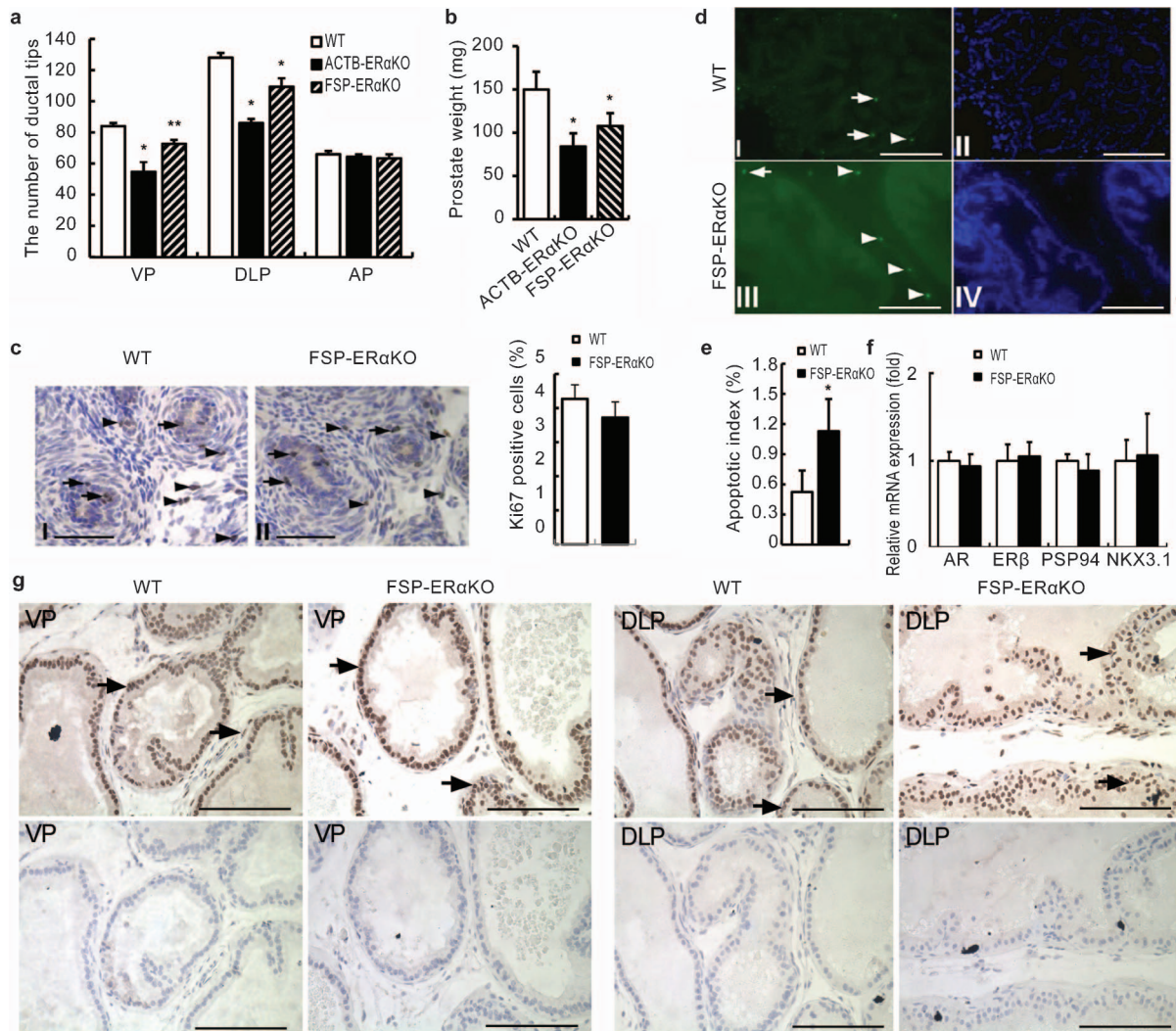
Figure 3 Loss of stromal ERα leads to the defective ductal morphogenesis, reduced prostate weight, and increased apoptosis. (**a**) The total number of ductal tips in ACTB-ERαKO, FSP-ERαKO and WT littermates of 14-week-old. There are significantly reduced ductal tips in VPs and DLPs, but not in APs, of both ACTB-ERαKO and FSP-ERαKO mice as compared to prostates of WT littermates. ***P<0.01, *P<0.001 *vs.* WT littermates (statistically significant by ANOVA analysis). Results are mean±s.d. (*n*=5). (**b**) The comparison of prostate weights of FSP-ERαKO and WT males at 3-month-old. *P<0.05 *vs.* WT. (**c**) The IHC detection of Ki67 expression in the VPs of FSP-ERαKO and WT males at 1-week-old. Arrows show positive staining of Ki67 protein in the epithelial cells. Arrowheads show positive staining of Ki67 in the stromal cells. The Ki67 proliferation index was scored as the percentage of Ki67-positive cells. (**d, e**) The apoptosis assay in the adult WT and FSP-ERαKO VPs. (**d**) The apoptosis activities are detected by TUNEL assay. Arrows show TUNEL-positive staining in the epithelial cells. Arrowheads show TUNEL-positive staining in the stromal cells. (**e**) The apoptotic index was scored as the percentage of TUNEL-positive cells. *P<0.05 *vs.* WT littermates (statistically significant by unpaired *t*-test). Results are mean±s.d. (*n*=4). (**f**) RT-PCR was used to compare the expression of AR, AR downstream target genes, and ERβ in adult FSP-ERαKO and WT littermates VP. Assays were performed on RNA from individual prostate lobes. The data are presented as mean±s.d. of samples collected from three different pairs of WT and FSP-ERαKO mice. Note that there is no difference in the mRNA expression of *AR, ERβ, PSP94* and *NKX3.1* between the adult VPs of FSP-ERαKO males and WT littermates. (**g**) IHC was applied to detect the AR expression in the adult FSP-ERαKO and WT littermate prostates. Arrows show positive AR staining in the epithelial cells. Scale bar is 100 μm. AP, anterior prostate; DLP, dorso-lateral prostate; ER, estrogen receptor; FSP, fibroblast-specific protein; IHC, immunohistochemistry; KO, knockout; RT-PCR, real-time PCR; T, testosterone; TUNEL, terminal transferase-mediated DNA end labeling; VP, ventral prostate; WT, wild type.

stromal proliferation (**Figure 3d**, arrowheads) between neonatal FSP-ERαKO and WT VPs. We also did not observe any significant proliferation difference between the adult FSP-ERαKO and WT prostates (data not shown). As there was no significant change in proliferative rate of neonatal and adult mouse prostate, we examined the apoptotic activities in the FSP-ERαKO and WT prostates. As expected, apoptosis occurred rarely in 1-week-old WT and FSP-ERαKO mouse prostates (data not shown). In adult mouse prostates, TUNEL assay indeed showed that there was a significantly increased stromal apoptosis in the FSP-ERαKO males (**Figure 3d**, III *vs.* I, arrowheads, VP data were shown), suggesting that the increased apoptotic rate contributed to the reduced

branching morphogenesis in the FSP-ERαKO mice. The quantitative apoptotic index is shown in **Figure 3e**.

The expressions of AR, AR target genes and ERβ are normal in the FSP-ERαKO prostates
It has been known that AR and ERβ could affect prostate development and homeostasis.[31–36] Therefore, we checked whether there were changes of AR and ERβ expression or functions in FSP-ERαKO prostates.

The expression of AR was detected *via* both IHC and Q-PCR methods. Our IHC results indicated that AR was located predominantly in the nuclei of luminal epithelial cells and there was no obvious

difference in AR expression levels or nuclear–cytosol distribution between adult FSP-ERαKO and WT VP and DLP (**Figure 3g**). Consistently, Q-PCR data suggested there was no significant change of AR mRNA in the FSP-ERαKO prostates (**Figure 3f**). AR target genes, *PSP94* and *NKX3.1*, markers of prostate cytodifferentiation, also expressed normally in FSP-ERαKO prostates as compared to WT littermates (**Figure 3f**). Furthermore, we did not observe any significant change in the expression of ERβ in the FSP-ERαKO prostates (**Figure 3f**). Together, these observations suggest that the defects of prostate ductal morphogenesis in the FSP-ERαKO prostates is not due to the alterations of serum T level (**Figure 2d**), or expressions of AR, AR target genes and ERβ (**Figure 3f** and **g**).

Generation and genotyping of pes-ERαKO mice

In addition to the stromal compartment, it is argued that prostate epithelial cells also express ERα and the epithelial ERα may contribute to the prostate development. To address this, it is important to use prostate epithelial cell-specific *Cre* to knock out the *ERα* gene. The similar breeding strategy of producing FSP-ERaKO mouse was applied to generate the pes-ERαKO mice (**Figure 4a**). The promoter of probasin is well characterized and widely applied in many animal models.[37–39] The expression of probasin *Cre* (PB-*Cre*) is prostatic epithelial-specific and is postnatal and gradually increased with highest expression in the lateral lobe, followed by the VP, and then the DP and AP.[37,38] Therefore, PB-*Cre* can specifically knock out the floxed *ERα* gene in prostate epithelial cells (pes-ERαKO).

To verify *ERα* gene deletion in the pes-ERαKO mice, young mice were genotyped for floxed ERα allele and PB-*Cre* transgene (**Figure 4b**). Since the *Cre* recombinase only expressed in the adult prostate epithelial cells, there is no ERαKO band detected in the tail snips of these pes-ERαKO mice (**Figure 4b**, lane 3). In 3-month-old pes-ERαKO mice, only the prostates revealed a recombined 223-bp DNA fragment of the ERα KO allele (**Figure 4c**, lane 4), indicating that PB-*Cre* selectively disrupts ERα in the prostates.

Although ERα protein expression is elevated in VP and DLP lobes in prostate cancer, or in estrogen-stimulated prostate proliferation and SQM, we and other investigators have found that ERα protein can be detected in VP and DLP, with a more stronger expression in AP of mice prior to any development of prostate diseases. Therefore, we compared the ERα expression in the AP of adult pes-ERαKO and WT littermates. ERα was mainly detected in the AP epithelial cells of WT mice (**Figure 4d**, left panel) and was rarely detected in pes-ERαKO AP (**Figure 4d**, right panel), which indicates that ERα is selectively ablated in pes-ERαKO prostate epithelial cells.

Genital tract phenotype, fertility and prostatic branching morphogenesis in adult pes-ERαKO males

Similar to FSP-ERαKO males, 3-month-old pes-ERαKO males did not exhibit any abnormalities in terms of the genital tract, sperm number and fertility (**Figure 5a–c**), suggesting that a postnatal depletion of epithelial ERα in the prostates did not affect the male reproductive system or fertility. Furthermore, pes-ERαKO prostates did not display any difference in weight and branching morphogenesis when compared to WT prostates (**Figure 5d** and **e**). Consistently, there was no significant changes in the expression of genes involved in prostatic branching morphogenesis (*BMP4, BMP7* and *Shh*), prostatic cytodifferentiation markers (*PSP94* and *NKX3.1*), or smooth muscle marker (*SM-α-actin*) between 3-month-old pes-ERαKO and WT prostates (**Figure 5f**). Further comparison between pes-ERαKO and WT
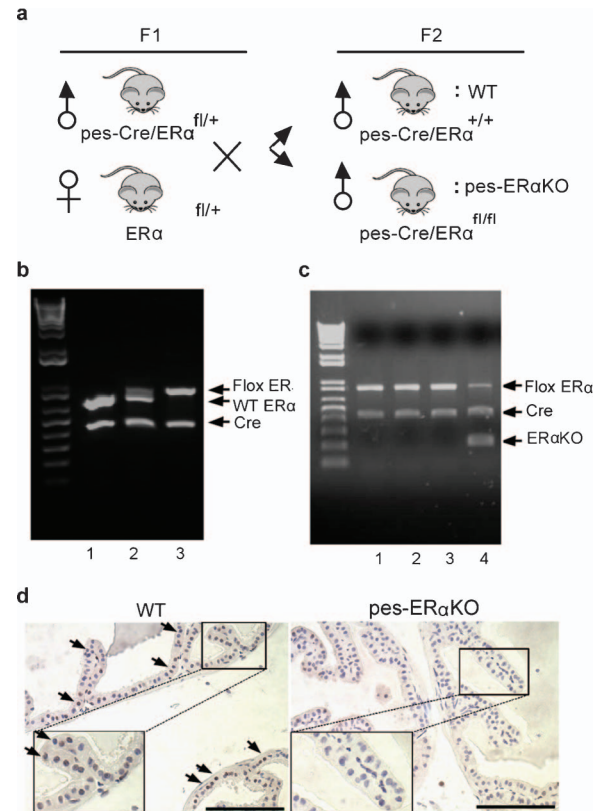

Figure 4 Generation and genotyping of pes-ERαKO male mice *via* Cre-loxP strategy. (**a**) Breeding strategy to generate the pes-ERαKO mice. ERα^{fl/fl} female mice were mated with probasin-Cre transgenic male mice to obtain the pes-Cre/ERα^{fl/+} male mice (F1). pes-Cre/ERα^{fl/+} male mice were mated with ERα^{fl/+} heterozygous female mice to obtain the pes-Cre/ERα^{+/+} (WT) and pes-Cre/ERα^{fl/fl} (pes-ERαKO) mice (F2). (**b**) Genotypes of tail snips of pes-Cre/ERα^{fl/fl} mice. Lane 1: pes-Cre/ERα^{+/+} mice, the size of WT ERα and Cre was 741 and 411 bp, respectively; lane 2: pes-Cre/ERα^{fl/+} mice; lane 3: pes-Cre/ERα^{fl/fl} mice. Probasin promoter will drive Cre specifically expressed in the prostatic epithelial cells in the adult stages, therefore, there is no KO allele present in the tail snips of pes-Cre/ERα^{fl/+} mice and pes-Cre/ERα^{fl/fl} mice (lanes 2 and 3). (**c**) Genotyping of various adult male reproductive organs of pes-ERαKO mice. Only the genomic DNA from the prostates of pes-ERαKO mice showed the ERαKO allele. Lane 1: testes; lane 2: epididymides; lane 3: efferent ducts; lane 4: prostates. (**d**) The expression of ERα in the APs of adult pes-ERαKO and WT was determined by IHC. Arrows show positive staining of ERα in the epithelial cells of WT mice. Scale bar is 100 μm. (Enlarged inserts are presented.) AP, anterior prostate; ER, estrogen receptor; IHC, immunohistochemistry; KO, knockout; pes-ERαKO, prostate epithelial cell-specific ERαKO; WT, wild type.

prostates was conducted on 12-month-old mice, since PB-*Cre* efficiency is believed to reach 100% at that age.[38] Comparisons revealed that pes-ERαKO and WT prostates have similar gene expression profiles and branching morphogenesis (data not shown). Taken together, our findings suggest that stromal ERα, but not epithelial ERα, is involved in prostatic branching morphogenesis and homeostasis of prostate.

Gene profile comparison in the WT, FSP-ERαKO and pes-ERαKO prostates

The prostatic branching morphogenesis is a complex interaction between prostate epithelial and mesenchymal cells regulated by time-specific and region-specific expression of signaling molecules.[40] Our previous studies found selective upregulation of BMP4 expression,
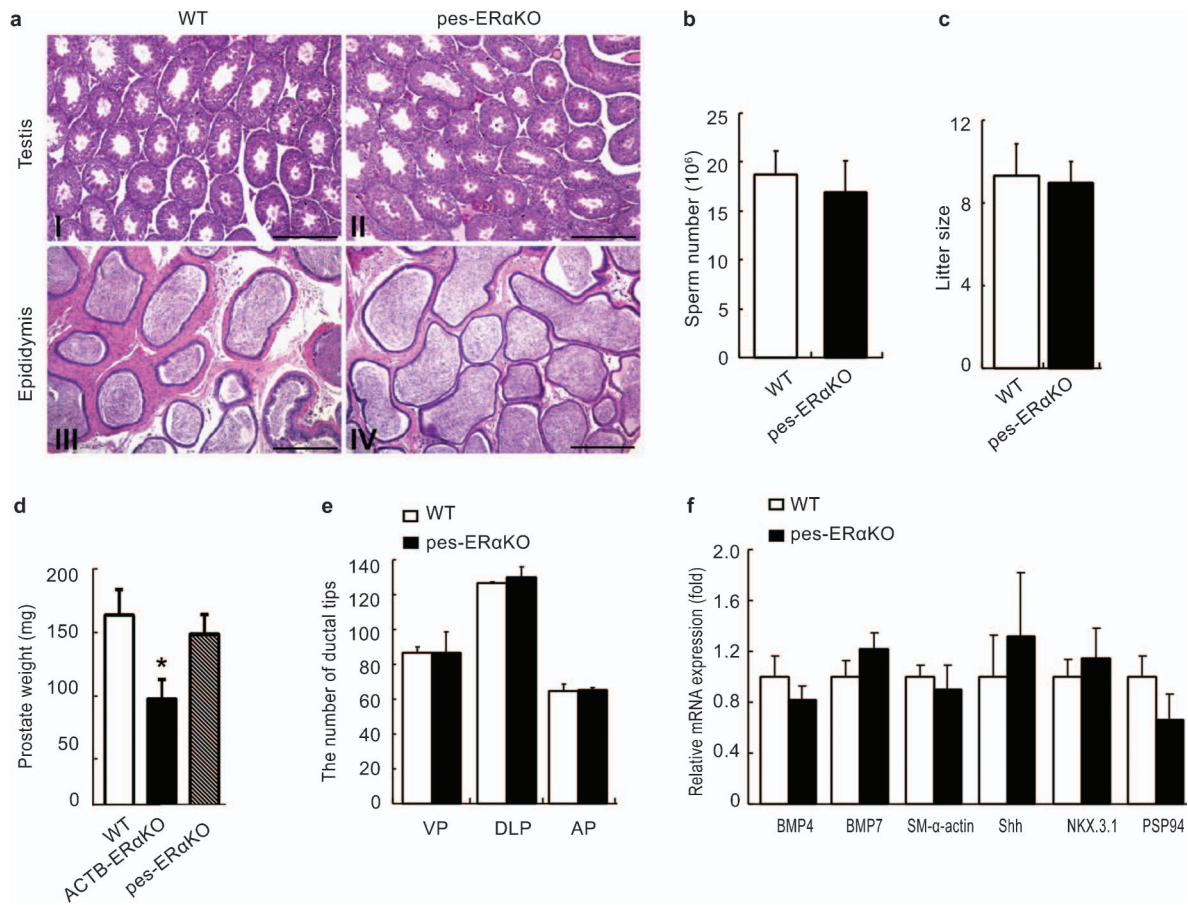
Figure 5 Histological analyses of testes and epididymides, fertility test and prostatic morphogenesis in the pes-ERαKO adult males. (**a**) Histological analyses of testes and epididymides from WT and pes-ERαKO males at 3-month-old. pes-ERαKO testes appeared normal and consisted of compact seminiferous tubules. Scale bar is 400 μm. The cauda epididymides from pes-ERαKO males contained sperm comparable to WT mice. Scale bar is 100 μm. (**b**) The comparison of the sperm count between adult pes-ERαKO and WT littermates. Results are presented as mean±s.d. (*n*=5). (**c**) Assess fertility of ERαKO males. Pup numbers per litter from WT females mating with WT or pes-ERαKO males were compared and found to be not significantly different. Results are presented as mean±s.d. (*n*=5). (**d-e**) The prostate weight and the total number of ductal tips in the pes-ERαKO and WT littermates shows no difference (*n*=5). *$P<0.05$ vs. WT littermates. (**f**) The comparison of the expression levels of the genes involved in prostatic branching morphogenesis and prostate differentiation between pes-ERαKO and WT littermates by real-time Q-PCR. Assays were performed on RNA from the VPs of each individual mouse and then the gene expression results were averaged. 18S rRNA was used as an internal control. Note that the genes involved in the ductal morphogenesis and prostate differentiation showed no significant difference between WT and pes-ERαKO mice. ER, estrogen receptor; FSP, fibroblast-specific protein; KO, knockout; pes-ERαKO, prostate epithelial cell-specific ERαKO; Q-PCR, quantitative PCR; VP, ventral prostate; WT, wild type.

but not BMP7, in the ACTB-ERαKO prostates.[18] As BMP4 is a mesenchymal factor that inhibits prostatic branching morphogenesis,[41] the upregulation of BMP4 in the ACTB-ERαKO prostates could directly contribute to the reduced branching morphogenesis.[18] As the reduced branching morphogenesis is also observed in the FSP-ERαKO mice, we were interested in determining whether BMP4 and other paracrine factors are regulated by prostate stromal ERα to influence branching morphogenesis. We examined the expression of several important genes involved in prostatic branching morphogenesis, including *sonic hedgehog* (*Shh*), the *fibroblast growth factor* (*FGF*) family, *insulin-like growth factor-1* (*IGF-1*), and the *bone morphogenesis protein* (*BMP*) family by RT Q-PCR. Our results showed that loss of stromal ERα in the mouse prostate leads to reduced expression of *IGF-1*, and increased expression of *BMP4* in the VP of FSP-ERαKO (**Figure 6a**, *$P<0.05$). There are no significant changes in the expression of *Shh*, *FGF7*, *FGF10* or *BMP7*, known to inhibit branching morphogenesis in the prostate.[42] In the pes-ERαKO, expression levels of these genes showed no significant difference. We also observed that expression of *IGF-1* decreases and *BMP4* increases in the DLP of

FSP-ERαKO (**Figure 6b**). Furthermore, FGF10 is reduced in DLPs of FSP-ERαKO as compared to those of pes-ERαKO and WT. However, we found *IGF-1* and *FGF7* expressions are slightly higher in the DLP of pes-ERαKO as compared to that of FSP-ERαKO. The slightly increased *IGF-1* and *FGF7* expressions may compensate for the *BMP4* growth inhibition effects in the prostates of pes-ERαKO mice. Together, our data suggested that there is a slightly different cytokine growth factor profile in the VP and DLP of ERαKO mice.

Collectively, the prostate branching morphogenesis can be regulated by inhibiting factors, such as BMP4, as well as promoting factors including *IGF-1* and *FGF10*. The current studies demonstrate that loss of stromal fibroblast ERα could alter the expression of important stromal factors that are involved in prostatic branching morphogenesis, and our results also suggest that stromal fibroblast ERα acts through a paracrine mechanism to regulate prostatic branching morphogenesis.

DISCUSSION

The prostate is a glandular structure consisting of epithelial and stromal compartments. A growing body of evidence has suggested that
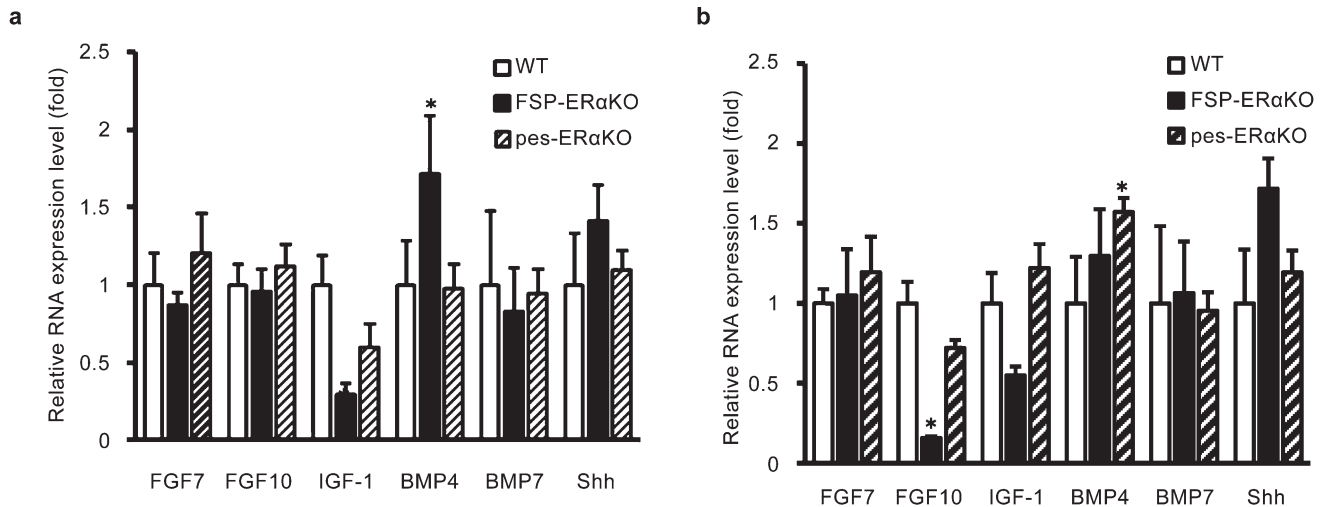
Figure 6 The genes regulated by stromal ERα in mouse prostate. Comparison of the expression levels of the genes involved in prostatic branching morphogenesis between FSP-ERαKO, pes-ERαKO and WT littermates by RT-PCR. Assays were performed on RNA from the VP (**a**) and DLP (**b**) of individual mouse and then averaged for the gene expression. *P<0.05 *vs.* WT littermates (n=4, statistically significant by unpaired *t*-test). β-actin was used as internal control. DLP, dorso-lateral prostate; ER, estrogen receptor; FSP, fibroblast-specific protein; IHC, immunohistochemistry; KO, knockout; pes-ERαKO, prostate epithelial cell-specific ERαKO; RT-PCR, real-time PCR; VP, ventral prostate; WT, wild type.

estrogen signaling exerts a direct impact on prostate pathogenesis and cancer.[18,21,43,44] Previous studies from conventional ER KO mice revealed that ERα, not ERβ, is the critical receptor to mediate the estrogens response in those processes.[7,8,19,21] To investigate the *in vivo* roles of ERβ in mouse prostate development, the neo-ERβKO mouse model was applied. The neo-ERβKO male mice from Chapel Hill revealed a hyperplastic prostate,[13] while the same transgenic mice housed at Karolinska Institute presented with prostatic intraepithelial neoplasia.[35] However, those phenotypes were not generally observed in the similar neo-ERβ mice generated by other groups.[45] Recently, a new and improved ERβKO mouse model has been developed in which exon III of the *ERβ* gene was deleted by *Cre*-loxP mediated excision and was devoid of any transcript downstream of exon III.[46] The studies using the *Cre*-loxP ERβKO mouse model showed that ERβ is not required for the development and homeostasis of the major body systems including the prostate, which further confirmed the important role of ERα, not ERβ, in the estrogen-mediated response in the prostate.

FSP-*Cre*-mediated excision of ERα could occur in multiple reproductive organs prenatally including the testes and epididymides (**Figure 1d**). Therefore, we examined whether there is any abnormality of fertility in the FSP-ERαKO. Compared to ACTB-ERαKO males, FSP-ERαKO males are fertile with normal development of testes and epididymides and serum T levels, which suggested that a prenatal deletion of stromal fibroblast ERα in the male reproductive organs does not affect male fertility. We also further delineated that loss of stromal fibroblast ERα in the mouse prostate glands resulted in reduced branching morphogenesis in FSP-ERαKO mice. FSP-*Cre* cannot completely knock out ERα in the stromal cells, and currently, there is no perfect stromal *Cre* mouse model available. However, FSP-*Cre* is known to knock down 50%–65% of floxed target genes in the mouse prostate,[44] which has been widely used to knock out a variety of target genes.[30,47] It is well documented that a low dose of estrogen can increase AR expression, prostate weight and prostatic budding in the rodent prostates,[48,49] which suggested that the physiological range of estrogens *via* ERs might promote prostatic branching morphogenesis and is consistent with our current studies that either loss of ERα in whole body[18] or only in stromal cells inhibit branching

morphogenesis in the rodent prostates. It is well known that there are endogenous estrogens in males. Fat cells can produce estrogen, T can be converted to E2 and DHEA can be converted by 17β DSH-1 to estrogenic compounds.[50] Therefore, results comparing WT, pes-ERαKO and FSP-ERαKO male prostates have provided the *in vivo* evidence that stromal ERα is important to regulate prostate branching morphogenesis. In addition, the high dose of estrogen will induce estrogen imprinting effect in neonatal prostate and SQM in the adult rodent prostates. We have recently published the characterization of the response of tissue-specific ERαKO prostates to high doses of estrogen.[44] From our studies, we found that in response to estrogen, FSP-ERαKO prostates develop a full and uniform SQM, In contrast, loss of epithelial ERα inhibits estrogen-mediated SQM evidenced by significantly decreased CK10 positive squamous cell stratification and differentiation, reduced upregulation of ERα expression, and the presence of normal proliferative activities in the estrogen-treated pes-ERαKO prostates. Together, these *in vivo* results suggest that stromal ERα is responsible for prostate branching morphogenesis and epithelial ERα is required for estrogen-mediated proliferative response and SQM development. Moreover, the branching morphogenesis observed between WT and FSP-ERαKO was significantly different. Our results indicated that phenotype changes are observed in VP and DLP, but not AP. The major reason may be that FSP-Cre was specifically expressed in the prostatic stromal fibroblast cells with strong expression in VP and DLP compared to AP (**Figure 1b**). Although the resulting phenotypes of arrested branching morphogenesis are similar to ACTB-ERαKO, the current study provides direct evidence that loss of ERα in the stromal fibroblast cells, but not in epithelial cells, of prostate reduces the formation of prostatic ductal tips and impairs branching morphogenesis. Moreover, in addition to BMP4 regulation of ERα, we also found that IGF-1 can be regulated by ERα in prostate fibroblast cells of intact mice *in vivo* and in primary cultured rUGM cells *in vitro*. Those stromal factors are specifically regulated by ERα, because genes in the same family, such as *FGF7* and *FGF10*, or other stromal factors are not affected by loss of stromal fibroblast ERα in the mouse prostate. IGF-1 has been considered as a growth factor and is well suited to act as a paracrine factor to

modulate mesenchymal–epithelial interaction in the prostate. The important roles of IGF-1 in normal prostate is revealed by the recent report that grafted prostatic tissue from IGF-1 KO and IGF-1R KO mice have retarded prostatic growth relative to grafts of normal urogenital sinus.[51] Our current studies emphasized IGF-1 could be involved in the prostatic development as a downstream gene regulated by stromal estrogen signaling in the prostate. Further studies are needed to determine the mechanism by which ERα regulates IGF-1 expression.

Our previous studies found that proliferation of prostate stromal cells was significantly reduced in ACTB-ERαKO mice at 1-week-old, which could partly contribute to the reduced branching in the ACTB-ERαKO prostates.[18] Interestingly, we did not observe any significant changes of proliferation rate in prostate epithelial and stromal cells of FSP-ERαKO compared to WT mice at 1-week-old (**Figure 3b**). Instead, we observed that the apoptotic index of FSP-ERαKO prostates was significantly higher than WT prostates (**Figure 3c** and **d**). The phenotypic difference in the prostates of ACTB-ERαKO and FSP-ERαKO mice suggested that ERα in stromal compartments may exert tissue or cell type-specific function and be involved in the prostatic stromal cell survival.

Probasin-Cre-mediated excision of ERα is highly specific in the prostatic epithelial cells (**Figure 5c**). As expected, pes-ERαKO males develop with normal fertility due to restricted Cre expression in prostatic epithelial cells. In contrast to FSP-ERαKO mice, the pes-ERαKO males develop normal prostates with normal ductal morphogenesis. The results suggest that postnatal deletion of epithelial ERα by probasin-Cre will not affect prostate homeostasis from fetal to adult stage. It has been well known that the mouse prostate undergoes extensive branching morphogenesis during the first 15 days of life.[30] Probasin promoter-driven *Cre* excision is barely detectable in the prostate during the first 15 days of neonatal mice,[37] which could be the reason that we did not observe the changes in the pes-ERαKO prostate development. In order to further define the role of epithelial ERα in the early prostatic development, the use of NKX3.1 *Cre* could be an ideal strategy, because its *Cre* activity begins to express at embryonic stage E17.5 in a prostatic epithelium-specific manner.[52] Alternatively, we could utilize other epithelial specific *Cre* mice, CK18-*Cre* and CK14-*Cre*,[53,54] to disrupt the *ERα* gene in the mouse prostate epithilium. In addition, Omoto *et al*.[16] has shown that the expression of ERα is stage-dependent and its expression in the mouse prostate epithelial cells could be changed from negative to positive under different challenges, such as estrogenic compound exposure,[44] castration followed by androgen supplementation[16] and pathophysiological progression from normal prostate to benign prostatic hyperplasia or prostate cancer. Therefore, it might be necessary to combine cell type-specific and temporal control *Cre* activity to knock out ERα in order to fully understand the roles of stromal *vs*. epithelial ERα in the prostate development, as well as prostate disease progression.[55]

In conclusion, we have generated prostate cell type-selective FSP-ERαKO and pes-ERαKO mouse models and have characterized the functions of ERα in the male genital tract and prostates using those two ERαKO mouse models. Our data support the hypothesis that ERα regulates the prostate in the context of stromal and epithelial interaction. The current studies using these tissue-specific KO mice extended our previous ACTB-ERαKO findings by validating and confirming that stromal fibroblast ERα plays an important role in prostate epithelial branching morphogenesis and prostatic homeostasis. Our study also demonstrated that there are several paracrine factors regulated by E2/ERα, and IGF-1 and BMP4 are responsible for E2/ERα signals in the prostate stromal cells.

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