Lactoferrin-iCre: A New Mouse Line to Study Uterine Epithelial Gene Function

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Transgenic animal models are valuable for studying gene function in various tissue compartments. Mice with conditional deletion of genes in the uterus using the Cre-loxP system serve as powerful tools to study uterine biology. The uterus is comprised of 3 major tissue types: myometrium, stroma, and epithelium. Proliferation and differentiation in each uterine cell type are differentially regulated by ovarian hormones, resulting in spatiotemporal control of gene expression. Therefore, examining gene function in each uterine tissue type will provide more meaningful information regarding uterine biology during pregnancy and disease states. Although currently available Cre mouse lines have been very useful in exploring functions of specific genes in uterine biology, overlapping expression of these Cre lines in more than 1 tissue type and in other reproductive organs sometimes makes interpretation of results difficult. In this article, we report the generation of a new *iCre* knock-in mouse line, in which *iCre* is expressed from endogenous lactoferrin (*Ltf*) promoter. *Ltf-iCre* mice primarily direct recombination in the uterine epithelium in adult females and in immature females after estrogen treatment. These mice will allow for specific interrogation of gene function in the mature uterine epithelium, providing a helpful tool to uncover important aspects of uterine biology. **(*Endocrinology* 155: 2718–2724, 2014)**

The uterus is a complex organ and has 3 major tissue compartments: myometrium, stroma, and epithelium. Coordinated actions of ovarian progesterone and estrogen regulate proliferation and differentiation of these uterine cell types in a spatiotemporal manner (1, 2). Disruption of this regulation leads to infertility and can initiate gynecological diseases, such as endometriosis, endometrial cancer, and many other conditions. More importantly, the uterine epithelium plays a critical role in embryo-uterine interactions for successful implantation. Currently, 4 Cre mouse lines are available for conditional inactivation of genes of interest in the uterus. Each of these Cre lines has its own unique uses, and they have been widely implemented to study gene function in the female reproductive tract (3–7).

Mice expressing Cre under the control of progesterone receptor (*PR*) (*PR-Cre*) have been widely used and have generated a wealth of information about gene function in uterine biology during pregnancy and in disease states (2). However, PR is expressed in all major uterine cell types, and thus, *PR-Cre* can delete floxed genes in all major uterine compartments (myometrium, stroma, and epithelium) (3, 6). Therefore, it is difficult to distinguish cell type-specific function of a gene if it is deleted in more than 1 cell type (8). In addition, PR is expressed in the oviduct, ovary, mammary gland, and pituitary. In this context, both oviductal and uterine defects were observed when a floxed *Tsc1* gene was deleted by *PR-Cre* (5). Females with conditional deletion of *Lgr5* by *PR-Cre* were also infertile, but the origin of defects was an ovarian failure to maintain progesterone secretion during pregnancy (9). Along the same line, anti-Müllerian hormone receptor type 2 (*Amhr2*)-*Cre* mice are commonly used to delete target genes in the stroma and myometrial compartments as well

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

Received March 26, 2014. Accepted May 5, 2014.
First Published Online May 13, 2014

Abbreviations: *Amhr2*, anti-Müllerian hormone receptor type 2; EGFP, enhanced green fluorescent protein; ES cell, embryonic stem cell; FRT, flippase recognition target; Ires, internal ribosomal entry site; lacZ, gene encoding β-galactosidase; *Ltf*, lactoferrin; *PR*, progesterone receptor; *PR-Cre*, Cre under the control of *PR*.

as in the ovary and oviduct (4–6). For deletion of genes of interest in the uterine epithelium, *Wnt7a-Cre* and *Sprr2f-Cre* mouse lines have been used. However, Cre activity under the control of the *Sprr2f* promoter is also observed in the cerebellum and kidney, and loxP recombination by *Sprr2f-Cre* in adult uterine epithelia is not uniform (7). *Wnt7a-Cre* deletes genes in the uterine epithelium and in epithelia of other organs, such as the hair follicular epithelium (see Supplemental Figure 2). Another unmet challenge in using *PR-*, *Amhr2-*, and *Wnt7a-Cre* mouse lines is that recombination occurs before the uterus is fully mature and in some cases affects uterine development (10–12), limiting the use of these lines in studying gene function in the adult uterus.

We sought to create a new mouse line for uterine epithelium-specific Cre recombination using a lactoferrin (*Ltf*) promoter (also called lactotransferrin). Ltf is a nonheme iron-binding glycoprotein, which is highly responsive to estrogen in the mouse uterus (13–17). *Ltf* is not expressed in immature mouse uteri, but it is robustly expressed in the uterine epithelium of adult mice (13, 15, 16). Furthermore, mice with constitutive deletion of *Ltf* are viable and fertile, albeit with minor alterations in iron homeostasis under normal conditions (18). In this study, we generated *Ltf-iCre* knock-in mice and show that iCre efficiently recombines floxed genes primarily in the uterine epithelium in adult females, and in immature females after estrogen treatment.

Materials and Methods

Targeting constructs

To create the *Ltf-iCre* knock-in targeting vector, the WI1–1277C21 fosmid that contains *Ltf* was acquired from the CHORI WIBR-1 mouse fosmid library and modified by a BAC recombineering method (19). A 25.5-kb region was removed from the 5′-end of the insert and replaced by a unique *Mlu*I site. Then, a 4.8-kb region at the 3′-end of the insert and a loxP site in the vector backbone were replaced by a diphtheria toxin fragment A expression cassette (20). As a result, the modified *Ltf* fosmid contains a 13.1-kb insert from the *Ltf* promoter to exon 8 of chr9: 111,012,552- 111,025,711 according to the December 2011 genome assembly (GRCm38/mm10) in the UCSC genome browser. Finally, an iCre-internal ribosomal entry site (Ires)-enhanced green fluorescent protein (EGFP)-flippase recognition target (FRT)-Neo/Kan-FRT cassette of pXL119 (a kind gift from Dr Gonzalo Alvarez-Bolado, Max Planck Institute, Munich, Germany) (21) with Kozak sequence replaced a part of exon 1 and intron 1 of *Ltf* at position chr9: 111,019,326- 111,019,369 in the GRCm38/mm10 genome assembly. The *Ltf-iCre* knock-in targeting vector was linearized by *Mlu*I for transfection.

Embryonic stem cell (ES cell) culture and gene targeting

The *Ltf-iCre* targeting construct was transfected into 129 and C57BL/6 hybrid G4 male ES cells (22) using a Bio-Rad Genepulser set at 500 μF and 0.24 kV. Neomycin-resistant cells were selected with 200 μg/mL G418, and ES colonies were picked 9–10 days after transfection. To confirm the targeted knock-in of *iCre* at the *Ltf* locus, Southern blot analyses were carried out. *Bam*HI- and *Eco*RI/*Xmn*I-digested genomic DNA were probed with probe 1 and probe 2, respectively, as shown in Figure 1A. Probes for Southern blotting were amplified from WI1–1277C21 by the following primers: probe1, Ltf5′P-F, GACATTCCTACTGCTCCTTGG and Ltf5′P-R, CCTGCTGTCCAGATGAGG; probe2, Ltf3′P-F, CACCAAGGACTGATGGATGA and Ltf3′P-R, TTCCATATTTTCCAAATGAACC. Thirteen homologously targeted ES clones were isolated out of 35 colonies picked (Figure 1B). Two *Ltf-iCre*-positive ES cell lines were microinjected into albino B6 mouse blastocysts to create mice (stock number 000058; The Jackson Laboratory). ES cell injection into blastocysts was performed at the Stem Cell

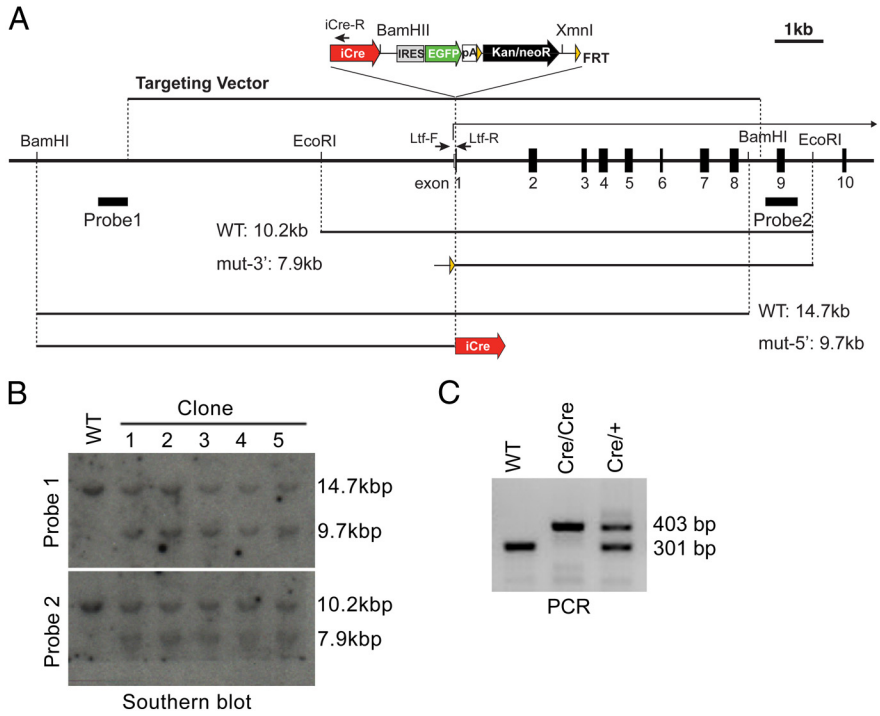

Figure 1. Generation of *Ltf-iCre* knock-in mice. A, Map of the *Ltf* genomic region. The *Ltf-iCre* gene targeting vector is shown above the map. B, Southern blot analysis of *Bam*HI-digested or *Eco*RI/*Xmn*I-digested gDNA with probe 1 or 2, respectively. C, Genomic PCR for genotyping using Ltf-F, Ltf-R, and iCre-R primers. Ltf-F and iCre-R primer set amplified a 403-bp fragment from the targeted *Ltf-iCre* locus, whereas a 301-bp fragment is amplified from wild-type *Ltf* by Ltf-F and Ltf-R primers. WT, wild type.

and Transgenics Core at Cornell University (Ithaca, New York).

Mice

The *Ltf-iCre* mouse line was generated and kept in a 129, C57BL/6 and albino B6 mixed background. To determine iCre localization and the efficiency of gene deletion, we mated them with B6.129S4-*Gt(ROSA)26*^{Sortm1Sor}/J (*R26R*$^{f/f}$) mice. Genotyping *Ltf*$^{iCre/+}$ mice was done by PCR using tail gDNA with primers: 5′-GTTTCCTCCTTCTGGGCTCC-3′ (Ltf forward), 5′-TTTAGTGCCCAGCTTCCCAG-3′ (Ltf reverse), and 5′-CCTGTTGTTCAGCTTGCACC-3′ (iCre reverse). The primers Ltf forward and Ltf reverse amplify a wild-type band (301 bp), and the primers Ltf forward and iCre reverse amplify a Ltf-iCre band (403 bp) (Figure 1C). Estradiol-17β (100 ng/mouse) was sc injected into *R26R*$^{f/f}$/*Ltf*$^{iCre/+}$ female mice 2 times (once daily at the ages of 21 and 22 d), which were killed at the age of 30 days to collect tissue for further analysis. All procedures for the present study were reviewed and approved by the Cincinnati Children's Research Foundation's Institutional Animal Care and Use Committee, in accordance with NIH guidelines.

LacZ (gene encoding β-galactosidase) staining

LacZ staining was performed as previously described (6, 23). In brief, tissues were embedded in optimum cutting temperature (OCT) compound after fixation in 0.2% paraformaldehyde and infusion in 30% sucrose at 4°C. Frozen sections were stained with 5-bromo-4-chloro-3-indolyl-β-d-galactopyranoside overnight at 37°C. The sections were counterstained with eosin.

Results

Generation of a new uterine epithelium-specific Cre mouse line

To create a uterine epithelium-specific Cre mouse line, we inserted iCre, which more efficiently deletes floxed genes under some experimental conditions as compared

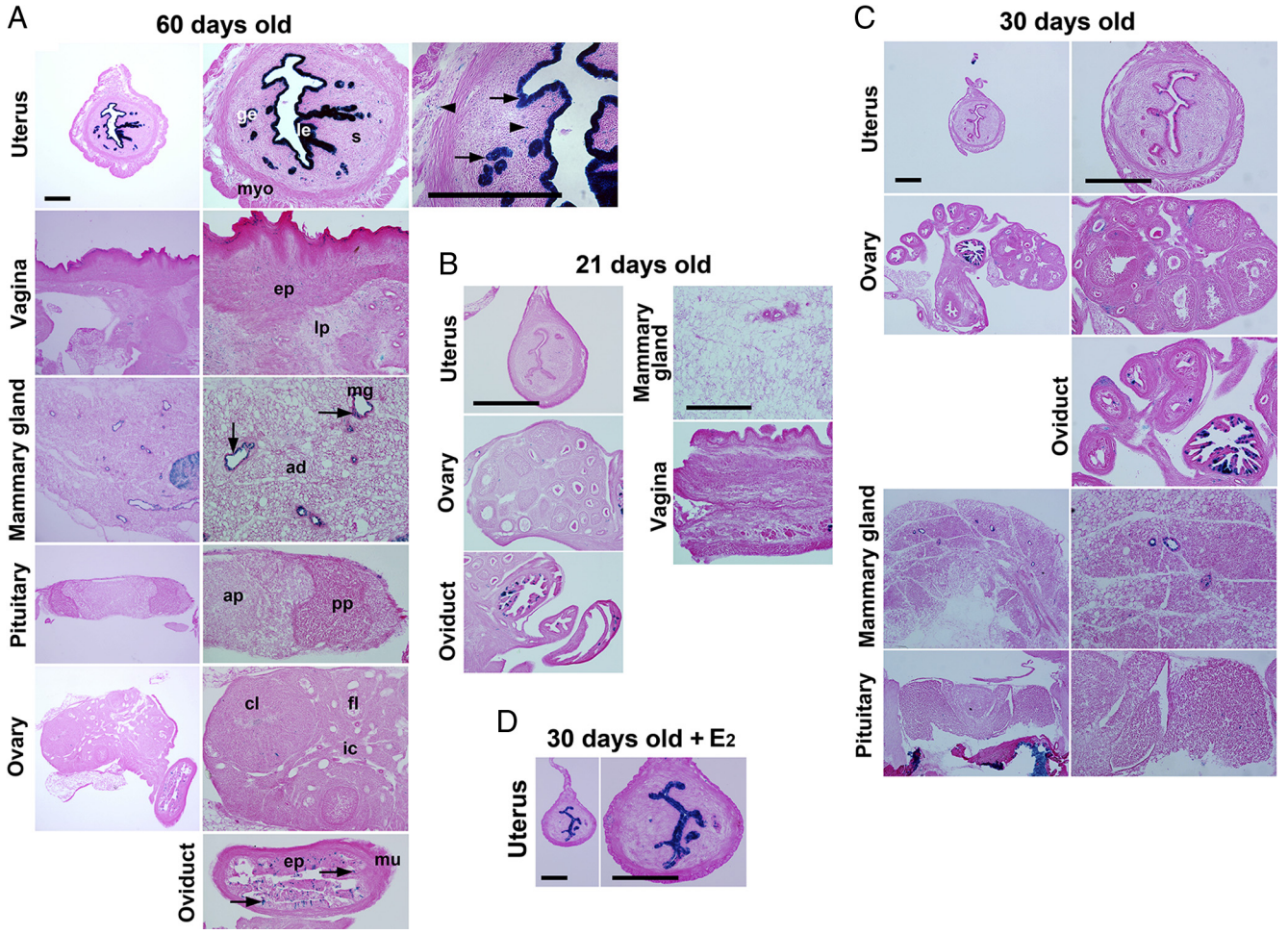

Figure 2. *Ltf*$^{iCre/+}$ efficiently recombines loxP sites in the uterine epithelium. Conditional gene recombination induced by *Ltf-iCre* was visualized by lacZ staining (A, 60 d; B, 21 d; C, 30 d; and D, 30 d old with estradiol-17β [E_2] treatment). Images in the left panels represent lower magnification, and those in the right panels are of higher magnification (A, C, and D). Arrows and arrowheads indicate lacZ-positive staining. Arrowheads depict lacZ-positive neutrophils in A. Scale bar, 400 μm. le, luminal epithelium; ge, glandular epithelium; myo, myometrium; s, stroma; ep, epithelium; lp, lamina propria; mg, mammary gland; ad, adipocyte; ap, anterior lobe of pituitary; pp, posterior pituitary; cl, corpus luteum; fl, follicle; ic, interstitial cell; mu, muscularis.

with standard Cre (24), under the control of the endogenous *Ltf* promoter by gene targeting (Figure 1). The iCre was followed by an Ires-EGFP to visualize *Ltf* promoter activity. Two *Ltf-iCre* targeted clones were used for blastocyst injection, and 8 chimeric male mice with 90%–100% contribution of *Ltf-iCre* ES clones as marked by coat color were selected for further analysis. Chimeras were bred with albino B6 mice twice to confirm germ line transmission. Male and female $Ltf^{iCre/+}$ mice were then mated to generate $Ltf^{iCre/iCre}$ mice. Breeding of male and female $Ltf^{iCre/+}$ mice produced normal litter sizes (7.1 ± 0.8, n = 12). Furthermore, breeding of $Ltf^{iCre/iCre}$ males and females also successfully produced pups (8.1 ± 1.0, n = 7). These results show that both $Ltf^{iCre/+}$ and $Ltf^{iCre/iCre}$ mice are viable and fertile. We also examined pregnancy timing with $Ltf^{+/+}$, $Ltf^{iCre/+}$, and $Ltf^{iCre/iCre}$ females mated with wild-type males. Parturition timing, litter size, and weight of pups of $Ltf^{+/+}$, $Ltf^{iCre/+}$, and $Ltf^{iCre/iCre}$ females were comparable (Supplemental Table 1). These results provide evidence that *iCre* expression under the *Ltf* promoter does not affect pregnancy outcome.

We next examined the efficiency of *iCre* recombination by crossing B6.129S4-*Gt(ROSA)26Sor*^{tm1Sor}/J ($R26R^{f/f}$) mice with $Ltf^{iCre/+}$ mice. $R26R^{f/f}$ mice induce β-galactosidase expression at the ROSA26 locus after Cre deletes a *polyA* signal flanked by loxP sites. As shown in Figure 2A, *Ltf-iCre* efficiently induced β-galactosidase expression in the uterine epithelium with nearly 100% efficiency as opposed to undetectable expression in the ovary and pituitary in mature $R26R^{f/f}/Ltf^{iCre/+}$ females. However, a limited population of cells in the oviduct, vagina, and mammary gland showed weak lacZ staining, and staining was also present in scattered neutrophils in the stroma and myometrium. The expression of *Ltf* in neutrophils in the uterine stromal bed was previously detected by in situ hybridization (16).

Ltf-iCre activity in immature mice

We next examined whether *Ltf-iCre* mediates floxed gene recombination in the uterus of immature mice (Figure 2, B and C). As expected, the number of lacZ-positive cells was very low to undetectable in the uterus at 21 and 30 days of age. LacZ staining was also detected in a subset of cells in the oviduct at the age of 21 days and in mammary glands at 30 days of age. To assess *Ltf-iCre* expression in the uterine precursor tissue in the fetal mesonephros, we examined *Ltf-iCre* activity in the Müllerian ducts on embryonic day 16 (vaginal plug = day 1). We crossed *Ltf-iCre* mice to B6;129S6-*Gt(ROSA)26Sor*$^{tm9(CAG-tdTomato)Hze}$/J mice,

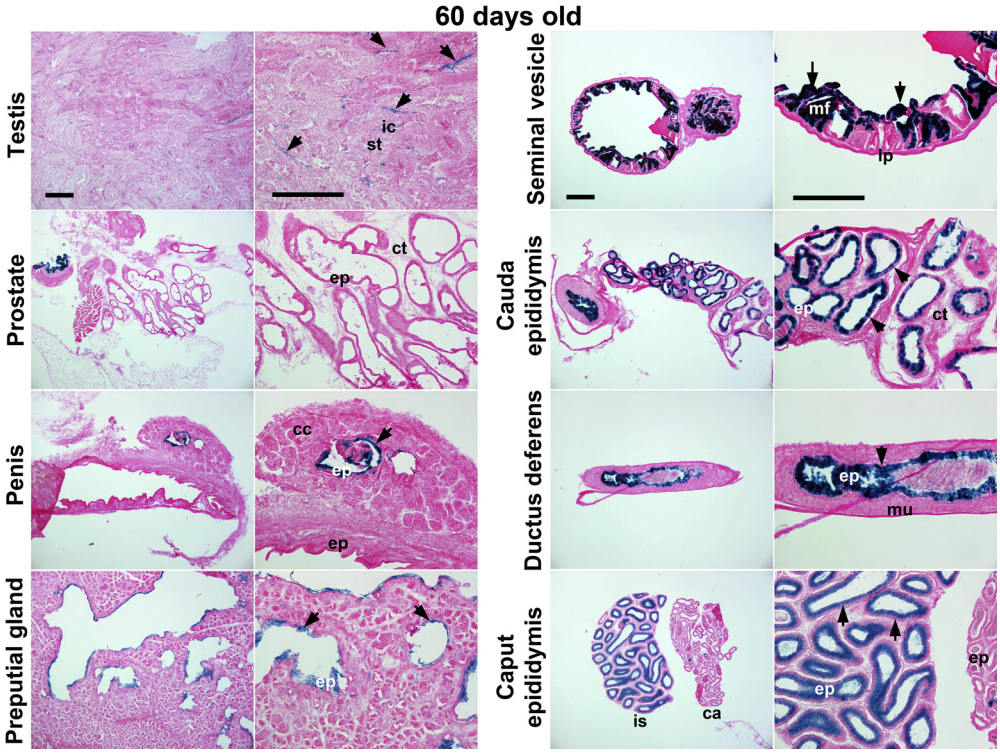

Figure 3. $Ltf^{iCre/+}$ induces recombination of loxP sites in male reproductive tissues. Conditional gene recombination induced by *Ltf-iCre* was visualized by lacZ staining. All samples are from 60-day-old males. Images in the left panels represent lower magnification, and those in the right panels are of higher magnification. Arrows indicate lacZ-positive staining. Scale bar, 400 μm. ic, interstitial cell; st, seminiferous tubule; ep, epithelium; ct, connective tissue; cc, corpus cavernosum; mf, mucosal fold; lp, lamina propia; mu, muscularis; is, intial segment of epididymis; ca, caput of epididymis.

which show tdTomato expression only after Cre-mediated recombination (due to *Ltf-iCre*-mediated excision of the *polyA* signal flanked by loxP sites) (Supplemental Figure 1). tdTomato expression was not detected in Müllerian ducts (marked by E-cadherin immunofluorescence), whereas it was detected in immune cells in the mesonephros; these are likely neutrophils, given that previous reports have demonstrated *Ltf* expression within neutrophils (16). These data suggest that *Ltf-iCre* does not induce loxP recombination in the fetal uterine precursor or in immature mice.

In contrast to *Ltf-iCre*, *PR-Cre* deleted floxed genes in all major uterine tissue compartments as early as 10 days of age (Supplemental Figure 2). *PR-Cre* also deleted floxed genes in the oviduct as early as 21 days old. In a similar manner, *Amhr2-Cre* deleted floxed genes in the stroma and myometrium in 10-day-old uteri, and also in the ovary and oviduct. *Wnt7a-Cre* deleted genes not only in uterine epithelia but also in the ovary and skin in 10-day-old mice. These results show that iCre expression under the regulation of the *Ltf* promoter generates more efficient Cre-loxP deletion primarily in the uterine epithelia of adult mice. Epithelial cell specificity of *Ltf-iCre* expression was also confirmed by examining EGFP expression from the iCre-Ires-EGFP cassette. Although EGFP expression was very low, but still detectable, without amplification of the signal by anti-GFP antibody staining, expression was observed in the uterine epithelium of mature mice (Supplemental Figure 3).

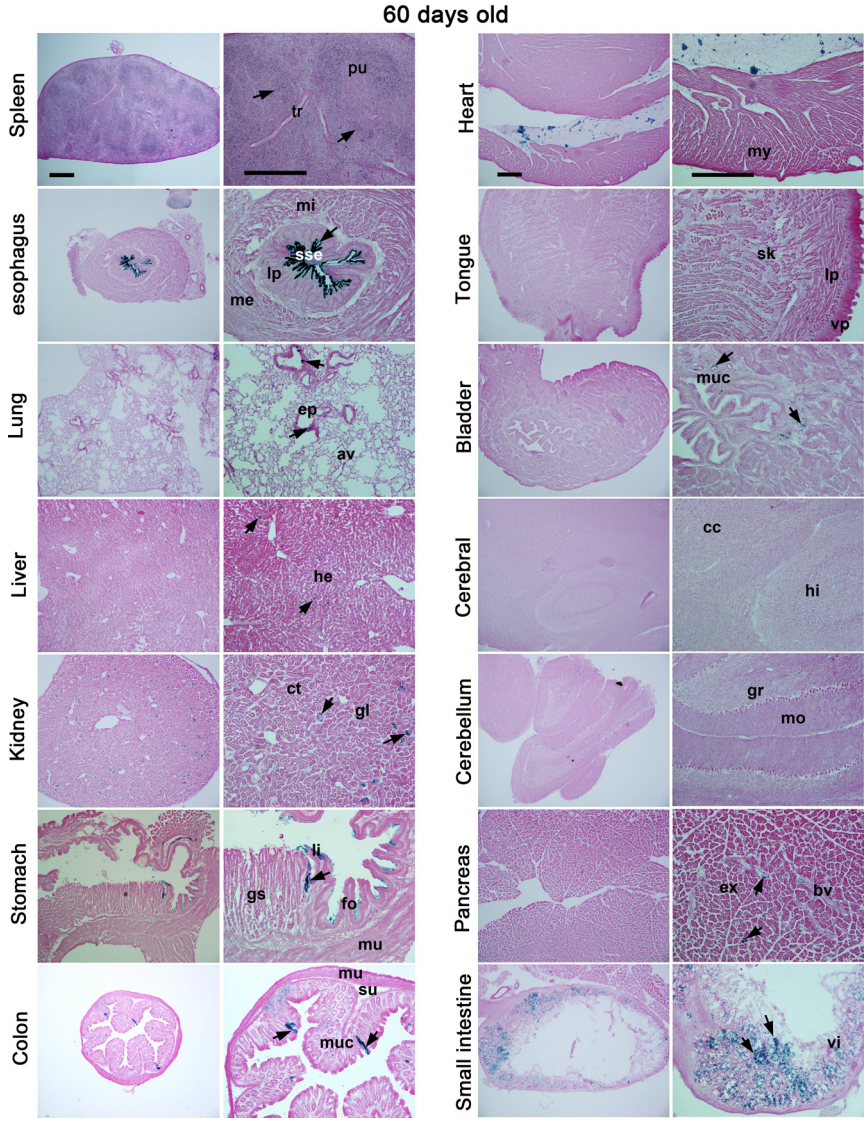

Figure 4. *Ltf*$^{iCre/+}$ shows limited recombination of loxP sites in other tissues. Conditional gene recombination induced by *Ltf-iCre* was visualized by lacZ staining. All samples are from 60-day-old females. Images in the left panels represent lower magnification, and those in the right panels are of higher magnification. Arrows indicate lacZ-positive staining. Scale bar, 400 μm. pu, pulp; tr, trabeculae; mi, muscularis interina; sse, stratified squamous epithelium; lp, lamina propria; me, muscularis externa; ep, epithelium; av, alveoli; he, hepatocyte; ct, convoluted tubule; gl, glomerulus; li, limiting ridge; gs, glandular stomach; fo, forestomach; mu, muscularis; su, submucosa; muc, mucosa, my, myocardium; sk, skeletal muscle; vp, ventral epithelium; cc, cerebral cortex; hi, hippocampus; gr, granular layer; mo, molecular layer; ex, exocrine pancreas; bv, blood vessel; vi, villus.

Because estrogen treatment can induce uterine epithelial *Ltf* expression in immature and mature mice (13, 14, 17), we examined whether *Ltf-iCre* can be induced by estrogen in immature mice. Indeed, we observed that estrogen treatment was effective in inducing *Ltf-iCre* activity in immature mice (Figure 2D). These results suggest that the estrogen-induced *Ltf* activation pathway is already present in immature mice, and Cre-loxP recombination can be induced by estrogen in immature *Ltf-iCre* mice before endogenous *Ltf* activation occurs.

Ltf-iCre activity in male reproductive tissues and other organs

We also examined Cre-loxP recombination in other tissues in *R26R*$^{f/f}$/*Ltf*$^{iCre/+}$ mice. Among male reproductive organs, some interstitial cells in the testis and epithelial cells in nontestis organs were lacZ positive (Figure 3). As shown in Figure 4, most tissues were negative or only a subset of cells was positive for lacZ expression except the esopha-

geal epithelium and spleen in $R26R^{f/f}/Ltf^{iCre/+}$ mice. Interestingly, lacZ-positive cells were also detected in the esophagus of $R26R^{f/f}/PR^{Cre/+}$ mice (Supplemental Figure 4). Because *Ltf* was reported to be expressed in preimplantation embryos (25), we examined Cre-loxP recombination in blastocysts using a double reporter mouse line (STOCK $Gt(ROSA)26Sor^{tm4(ACTB-tdTomato,-EGFP)Luo}$/J) (Supplemental Figure 5). This mouse line expresses tdTomato in all tissues, but tdTomato expression is switched to EGFP expression when Cre-loxP recombination occurs. Because EGFP expressed from Ltf-iCre-Ires-EGFP was undetectable (Supplemental Figure 5, bottom panels), we expected that any detectable EGFP signal would originate from conversion of tdTomato to EGFP expression mediated by iCre. However, we failed to detect EGFP fluorescence in blastocysts obtained from these mice. This suggests that iCre driven by the *Ltf* promoter is not active in blastocysts.

Discussion

We present here the generation of *Ltf-iCre* mice that show efficient and specific deletion of floxed genes in the uterine epithelium, which could be of great value in studying uterine epithelial gene function during pregnancy and in immature mice after hormonal stimulation. A variable, but generally low, level of *Ltf-iCre* activity was also observed in other tissues, such as male reproductive organs, neutrophils, spleen, liver, kidney, and epithelia of esophagus, lung, intestine, and bladder. Although the activity of *Ltf-iCre* was strongest and most consistent in the uterine epithelium, its expression was higher in neutrophils, spleen, and esophagus than the other nonuterine tissues, in which subsets of cells were lacZ positive. Thus, as with most Cre lines, some caution is raised as to the deletion of a target gene that may occur in other undesired tissues, potentially resulting in unexpected phenotypes. However, *Ltf-iCre* showed nearly 100% efficiency of recombination in the uterine epithelium, starting only in adult stages between 1 and 2 months of age. Additionally, we found very low to undetectable activity in the pituitary and ovary. Therefore, this Cre line will provide a new way to assess gene function specifically in the adult uterine epithelium, with perhaps relatively fewer concerns about confounding effects of gene deletion in other components of the reproductive tract or hormonal machinery.

It may also be worthwhile to examine whether *Ltf* deletion in the *Ltf-iCre* mice produces any phenotype, because *Ltf* has roles in defense against oxidative stress and infection and has immunomodulatory function under certain conditions (26, 27). However, *Ltf* knockout mice show a mild phenotype, with only minor alterations in iron homeostasis under normal conditions (18). We believe that using *Ltf-iCre* mice to induce conditional targeting of floxed genes in the uterine epithelium should not pose any appreciable problem, because the recombination of the target gene can be efficiently achieved with heterozygous *Ltf-iCre* alleles; 1 allele of *Ltf* remains intact to allow for normal immunomodulatory function. This new *Ltf-iCre* mouse line is likely to be a powerful tool to define the contribution of specific genes in uterine epithelial cell function under normal and abnormal uterine environments.

Acknowledgments

We thank Serenity Curtis for editing the manuscript. The iCre-Ires-EGFP-FRT-PGK-neo-FRT construct was kindly provided by Gonzalo Alvarez-Bolado (University of Heidelberg, Heidelberg, Germany). The G4 ES cell line was provided by Andras Nagy and Marina Gertsenstein (Mount Sinai Hospital, Canada). ES cell injection into blastocysts was performed by Christian Abratte and Robert Munroe (Stem Cell and Transgenics Core, Cornell University, Ithaca, NY). *PR-Cre* mice were initially provided by Francesco DeMayo and John B. Lydon (Baylor College of Medicine, Houston, TX). *Amhr2-Cre* and *Wnt7a-Cre* mice were provided by Richard Behringer (MD Anderson Cancer Center, Houston, TX).

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This work was supported in part by National Institutes of Health Grants HD068524, DA06668, and PO1CA7783 and by March of Dimes (S.K.D.). J.T. is supported by a Japan Society for the Promotion of Science postdoctoral fellowship sponsored by Kyoto University (Kyoto, Japan) and Cincinnati Children's Hospital. Stem Cell and Transgenics Core, Cornell University is supported by New York State Department of Health Contract C024174.

Disclosure Summary: The authors have nothing to disclose.

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