SHORT COMMUNICATION

Utility of progesterone receptor-ires-Cre to generate conditional knockout mice for uterine study

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Abstract

In mice, the conditional knockout strategy using the Cre-loxP system is useful for various types of research. The Cre mouse line with progesterone receptor promoter (Pgr^{Cre}) has been widely used to produce specific uterine gene-deficient mice, but in the Cre line, endogenous Pgr gene is replaced by Cre recombinase gene, which makes the breeding of homozygous mice ($Pgr^{Cre/Cre}$) difficult because they are infertile. Yang et al. (2013, https://10.1016/j.cell.2013.04.017) reported the generation of another $Pgr^{iresCre}$ mouse line that still has endogenous Pgr gene, and they inserted Cre recombinase downstream of the Pgr gene via an internal ribosome entry site (IRES). It is possible that this new $Pgr^{iresCre}$ line would be useful for uterine research as the mice can be bred as homozygotes ($Pgr^{iresCre/iresCre}$). Herein, we confirmed the $Pgr^{iresCre}$ mice effectively directed recombination in the female reproductive tract and was capable of genetic alteration in the endometrium that enables the studies of its uterine function. Our findings demonstrate that the new $Pgr^{iresCre}$ mouse line is also useful for the generation of uterine-specific knockout mice. The findings using $Pgr^{iresCre}$ mouse will contribute to the understanding of reproductive systems and diseases in humans and domestic animals.

KEYWORDS
Cre-loxP system, progesterone receptor, uterine function, β-galactosidase (β-gal) staining

1 | INTRODUCTION

Genetic modification technologies in mice have advanced dramatically in recent years (Lee et al., 2020). The Cre-loxP system in particular, which is derived from bacteriophage P1, is an indispensable tool for cell type-specific gene editing, including target gene activation and inactivation (Huang et al., 2017; Kim et al., 2018). This system is based on the mechanism in which Cre recombinase recognizes the specific 34-base pair sequence called loxP, and the orientation and location of the loxP sites determine how the genetic rearrangement will be achieved. If two loxP sites with the same orientation are inserted into specific DNA sequences, the target sequences between two loxP sites (which are called "floxed") are eliminated in a Cre-dependent manner. The Cre-loxP system has thus enabled researchers to regulate genes' expression or repression in a tissue- or cell-specific (spatial control) manner and/or in a time-specific (time control) manner. The conditional knockout strategy is a powerful tool when the inactivation of target genes causes embryonic lethality or growth failure in cases of constitutive knockout.

To control the expression of Cre, various tissue/cell-specific Cre-driver mouse strains have been generated so that Cre recombinase is expressed under the promoter that specifically targets the tissue or cell of interest (Korecki et al., 2019; Mattiuz et al., 2018). The Cre-driver mouse under the progesterone receptor (*Pgr*) promoter (*Pgr^Cre*) generated by Soyal et al. (2005) has been widely used in the study of uterine biology, as PGR is widely expressed in uterine cellular compartments, that is, the luminal epithelium, glandular epithelium, stroma cells, and myometrium. PGR, which is activated by progesterone, is a member of the nuclear receptor superfamily and composed of two primary isoforms (the full-length PGR-B and N-terminal-truncated PGR-A) derived from one gene (Bain et al., 2000). The PGR null mice are sterile due to multiple defects in the reproductive tissues, including an inability to ovulate, uterine hyperplasia, and impaired mammary development (Lydon et al., 1995). In the *Pgr^Cre* mouse, the first exon of endogenous *Pgr* gene is replaced with *Cre* recombinase gene, which makes the breeding of homozygous mice (*Pgr^{Cre/Cre}*, the same as *Pgr* null) difficult. Although many fundamental genes that are essential for uterine function have been identified by using *Pgr^Cre* mice (Matsumoto et al., 2018; Namiki et al., 2018; Vasquez et al., 2018), the potential negative effect(s) on the phenotype due to the loss of one allele of *Pgr* gene has not been considered in the conditional knockout mice.

Yang et al. (2013) reported the generation of another Cre-driver mouse strain in which *Cre* recombinase gene is inserted downstream of the *Pgr* gene combined with an internal ribosome entry site (IRES) (*Pgr^{iresCre}*), leading to the expression of both endogenous *Pgr* and exogenous *Cre*. This new type of *Pgr^{iresCre}* mouse is likely to be more useful for uterine research; in addition, the homozygous females of the Cre line (*Pgr^{iresCre/iresCre}*) are fertile (Yang et al., 2013). Successful genetic alteration has been reported in the PGR-expressing cells by the combination of floxed mouse with *Pgr^{iresCre}* mouse (Yang et al., 2013). Xin et al. (2018) have already utilized *Pgr^{iresCre}* mouse for uterine Cre-loxP recombination in their study; however, it remains unclear how efficiently this *Pgr^{iresCre}* can alternate genetic recombination in the endometrium.

In this study, we investigated the Cre expression pattern in the female reproductive tissues of *Pgr^{iresCre}* mice by using a reporter strain (*Rosa26^{NLSLacZ}*). We also evaluated whether *Pgr^{iresCre}* can ablate floxed genes to generate uterine-specific conditional knockout mice. We targeted *Stat3* and *Gp130* (also known as *Il6st*) in the endometrium, as both of them play an important role in establishment of embryo implantation by mediating leukemia inhibitory factor (LIF) signal, and either genetic recombination with *Pgr^Cre* exhibits female infertility (Sun et al., 2013).

2 | MATERIALS AND METHODS

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise stated. All animal procedures were approved by the Ethical Committee for Vertebrate Experiments at Azabu University (ID#200312-24).

2.1 | Animals

Animals were housed in the barrier facility at Azabu University. The following mouse strains were used: *Pgr^{iresCre/+}* mouse <B6.129S(Cg)-*Pgr*tm1.1(cre)Shah/AndJ, Stock No: 017915> (Yang et al., 2013) purchased from Jackson Laboratory (Bar Harbor, ME, USA), *Rosa26^{NLSLacZ/+}* mouse <B6.129P2-Gt (ROSA)26Sor<tm1 (NLS-lacZ)Ito> (BRC02657) provided by RIKEN BRC through the National Bio-Resource Project of the MEXT/AMED (Ibaraki, Japan) (Kobayashi et al., 2013), *Stat3^{flox/flox}* (*Stat3^{f/f}*) mouse <Stat3^{tm2Aki}> (Takeda et al., 1998), and *Gp130^{flox/flox}* (*Gp130^{f/f}*) mouse <Il6st^{tm1Wme}> (Betz et al., 1998). All mice were fed ad libitum under a 12-h light/12-h dark photocycle at $23 \pm 2^\circ$C.

For our investigation of the expression of Cre-dependent *lacZ* (gene coding β-galactosidase [β-gal]), we generated *Pgr^{iresCre/+}*;*Rosa26^{NLSLacZ/+}* mice. Four-, 8-, and 12-week-old *Pgr^{iresCre/+}*;*Rosa26^{NLSLacZ/+}* females were sacrificed, and reproductive tissues were collected for β-gal staining. Before the evaluation of the β-gal activity in the mouse ovaries, hormonal treatments for hyper-ovulation were performed by an intraperitoneal injection of equine chorionic gonadotropin (eCG, 5 IU; Nippon Zenyaku Kogyo, Fukushima, Japan) followed by human chorionic gonadotropin (hCG, 5 IU; Nippon Zenyaku Kogyo) as described (Kohaya et al., 2013). Pregnant reproductive tissues were collected at the indicated pregnancy day(s) after mating with fertile males. Day 1 of pregnancy was defined as the day on which a vaginal plug was first observed.

We generated uterine-specific gene knockout mice by crossing *Pgr^{iresCre/iresCre}* male mice with *Stat3^{f/f}* or *Gp130^{f/f}* female mice, and uterine-specific gene knockout female mice were mated with fertile males to check their fertility. Implantation sites at Day 5 of pregnancy were identified by a tail-vein injection of Chicago blue dye as described (Kelleher et al., 2017).

2.2 | β-gal staining

β-gal (coded by *LacZ*) staining was performed as described (Daikoku et al., 2014; Soyal et al., 2005). In brief, reproductive tissues from female *Pgr^{iresCre/+}*;*Rosa26^{NLSLacZ/+}* were fixed with prechilled 0.2% paraformaldehyde (PFA) in phosphate-buffered saline (PBS), 5-mM ethylene glycol tetra acetic acid (EGTA), and 2-mM $MgCl_2$ at 4°C overnight. The tissues were washed with 2-mM $MgCl_2$ in PBS three times, followed by equilibration to 30% (w/v) sucrose with 2-mM $MgCl_2$ in PBS. The tissues were then embedded in optimum cutting temperature (OCT) compound (Sakura Finetek, Tokyo, Japan) and stored at -80°C.

Frozen 16-μm sections cut by a cryostat were stained with 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) and maintained at 37°C overnight. The sections were counterstained with eosin. The whole-mount staining was performed by the same procedure as that used for the frozen sections, with the exception of the cutting step.

3 | RESULTS

3.1 | β-gal expression in the female reproductive tissues

To investigate whether *Pgr*^{iresCre} mediates a floxed gene recombination in the female reproductive tissues of mice, we crossed *Pgr*$^{iresCre/}$ ^{iresCre} mice with *Rosa26*$^{NLSLacZ/NLSLacZ}$ mice to generate *Pgr*$^{iresCre/+}$;*Rosa26*$^{NLSLacZ/+}$ mice, which induces Cre-dependent nuclear *LacZ* (coding β-gal) expression. We observed β-gal expression (visualized in blue) in all of the major compartments of the uterine tissue of mice from 4 to 12 weeks old, including the luminal epithelium, glandular epithelium, stroma cells, and myometrium (Figure 1a). β-gal activity was also detected in the oviduct (muscularis layer and mucosa layer) (Figure 1b). Scattered β-gal staining was observed in some theca/interstitial cells of the ovary from 4-week-old mice and in the corpus luteum after sexual maturation at 8 weeks old (Figure 1b).

PGR expression is induced in granulosa cells of the preovulatory follicles after hormonal treatment for hyperovulation (Ismail et al., 2002). We thus collected female reproductive tissues at 0, 12, 24, and 48 h after the injection of hCG primed by eCG. Strong β-gal staining was visible in the oviduct and the uterus by whole-mount staining (Figure 2a). The β-gal staining was gradually increased in the corpus luteum from 12 to 24 h after the hormonal treatment as shown by the whole-mount staining (Figure 2a). In the cross sections, β-gal expression was detected in some theca/interstitial cells and luteal cells, but not in granulosa cells in growing and preovulatory follicles (Figure 2a).

We also assessed the β-gal expression in the early pregnant uterus from Days 1 to 8, and we observed that β-gal was constitutively expressed in uterine cells in addition to the decidual cells on Day 8 (Figure 2b). This result demonstrates that *Pgr*^{iresCre} effectively mediates the floxed gene recombination in the whole endometrium.

3.2 | Utility of *Pgr*^{iresCre} mouse in generating conditional knockout

To determine whether the *Pgr*^{iresCre} strain is useful for uterine study, we generated conditional knockout mice as *Pgr*$^{iresCre/+}$;*Stat3*$^{f/f}$ (*Stat3*$^{d/d}$) mice or *Pgr*$^{iresCre/+}$;*Gp130*$^{f/f}$ (*Gp130*$^{d/d}$) mice. Both Stat3 and Gp130 are pivotal for the embryo implantation to mediate indispensable signaling from leukemia inhibitory factor (LIF) (Sun et al., 2013), and both constitutive knockout strains are embryonic lethal (Betz et al., 1998; Takeda et al., 1997). The numbers of offspring (average ± SEM) were 6.7 ± 0.6 (*Stat3*$^{f/f}$) and 8.0 ± 0.5 (*Gp130*$^{f/f}$) in the control groups, whereas neither the *Stat3*$^{d/d}$ females nor the *Gp130*$^{d/d}$ females gave birth (Table 1). No implantation site was observed in the uterus of either conditional knockout line, and unimplanted blastocysts were
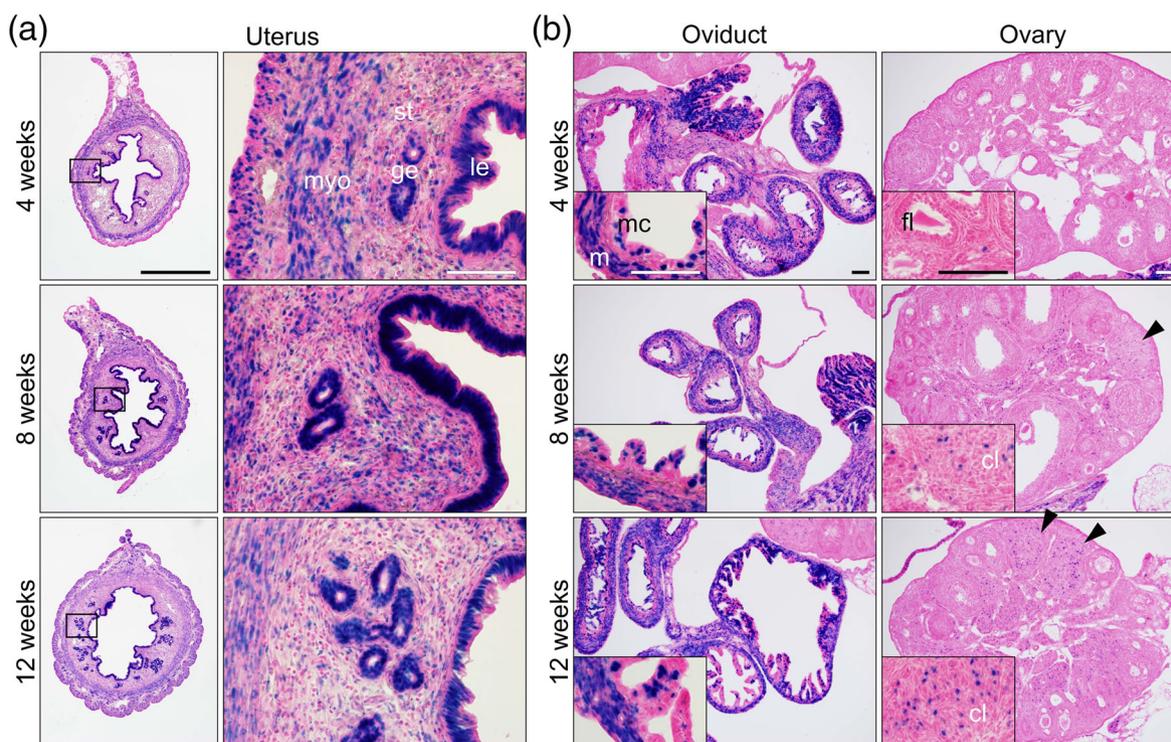

FIGURE 1 *Pgr*^{iresCre} recombines loxP sites in the female reproductive tracts. The genetic modification induced by *Pgr*^{iresCre} was visualized by β-galactosidase (β-gal) staining (4, 8, and 12 weeks old). (a) Images from the uteri. Left panels represent lower magnification (scale bar, 1 mm), and those in the right panels are of higher magnification of indicated area in the left panels (scale bar, 100 μm). (b) Images from the oviducts and ovaries. Higher magnification of the images is inserted in the lower left. Black arrows indicate the corpus luteum that contains β-gal-positive cells. Scale bars, 100 μm. cl, corpus luteum; fl, follicle; ge, glandular epithelium; le, luminal epithelium; m, muscularis layer; mc, mucosa layer; myo, myometrium; st, stroma
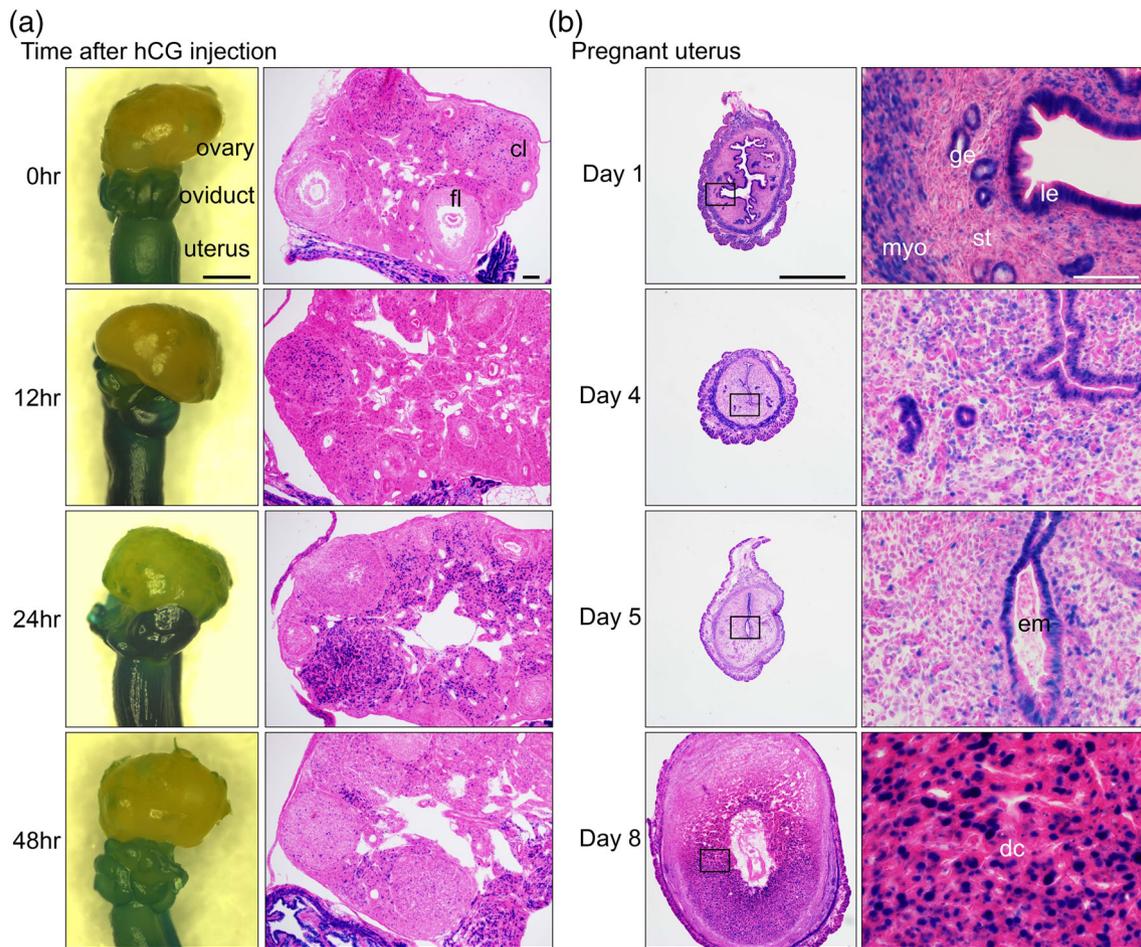
FIGURE 2 Cre activities in $Pgr^{iresCre}$ mice after hormonal treatment and during early pregnancy. (a) Cre activity is visualized by whole mount β-galactosidase (β-gal) staining 0, 12, 24, and 48 h after human chorionic gonadotropin (hCG) injection primed by equine chorionic gonadotropin (eCG) (left panels; scale bar, 1 mm). Right panels represent the cross sections of ovaries (scale bar, 100 μm). (b) Cre activity in the $Pgr^{iresCre}$ uteri during early pregnancy. Left panels represent lower magnification (scale bar, 1 mm), and those in the right panels are of higher magnification of indicated area in the left panels (scale bar, 100 μm). cl, corpus luteum; dc, decidual cells; em, embryo; fl, follicle; ge, glandular epithelium; le, luminal epithelium; myo, myometrium; st, stroma

TABLE 1 Pregnancy failure in the conditional knockout of $Stat3$ or $Gp130$ with $Pgr^{iresCre}$

Genotype	No. of females examined	Total number of litters	Litter size (mean ± SEM)
$Stat3^{f/f}$	7	47	6.7 ± 0.6
$Stat3^{d/d}$	15	0	0
$Gp130^{f/f}$	6	48	8.0 ± 0.5
$Gp130^{d/d}$	11	0	0

recovered from the uterine horn at Day 5 of pregnancy in both lines, indicating that both $Stat3^{d/d}$ females and $Gp130^{d/d}$ females are infertile due to the failure of embryo implantation.

4 | DISCUSSION

The results of this study demonstrated that $Pgr^{iresCre}$ is useful to rearrange floxed genes in PGR-expressing cells of the mouse uterus. Because Cre-loxP excision is irreversible, a genetic modification in PGR-expressing cells is inherited by daughter cells once the Cre-mediated recombination is achieved in the mother cells. The expression of PGR in the mouse uterus is first observed by immunohistochemistry in the luminal epithelium on postnatal Day 5 (Kurita et al., 2004), and the expression is subsequently increased in the subepithelial stroma and myometrium as the mouse matures (Soyal et al., 2005). PGR expression in the uterus is regulated by ovarian steroids, that is, progesterone and 17β-estradiol, leading to changes in the PGR expression during the estrus cycle and pregnancy (Kurita et al., 2000; Tan et al., 1999).

Our present findings revealed that the age of 4 weeks in *Pgr^iresCre* mice (at which time they are still sexually immature) is a sufficient age for the observation of rearranged floxed genes in the entire uterus. An effective genetic alteration in reporter strains with *Pgr^iresCre* was also confirmed in sexually mature uterine samples and early-pregnancy samples. In fact, the combination of *Pgr^iresCre* with *Stat3^f/f* or *Gp130^f/f* resulted in the same embryo implantation defect phenotype as that reported in a study using *Pgr^Cre* mice (Sun et al., 2013). Xin et al. (2018) have reported that the genetic recombination of their target gene by *Pgr^Cre* mice or *Pgr^iresCre* mice showed similar uterine impairments. We confirmed that *Pgr^iresCre* mice can be used for targeting uterine tissues.

The oviduct is also a target tissue of Cre-mediated genetic alteration under *Pgr* promoter. The isoform PGR-B is dominantly expressed in the epithelial cells of the oviduct, whereas PGR-A is expressed mainly in the stromal and smooth muscle cells (Gava et al., 2004). High β-gal expression was detected only in epithelial cells of the oviduct in Soyal et al. (2005) study of *Pgr^Cre* with a reporter strain. In contrast, in the present study, high β-gal expression was observed in the stromal and smooth muscle cells of the oviduct as well as epithelial cells. This discrepancy is most likely due to the different regulation of *Pgr* gene isoforms because PGR-B and PGR-A are encoded by a single *Pgr* gene and use two distinct promoters (Kastner et al., 1990). Cre expression in *Pgr^Cre* represents PGR-B expression because *Cre* recombinase gene is inserted into exon 1 of the *Pgr* gene and translational start sites of PGR-A is eliminated by this insertion (Soyal et al., 2005). Although *Cre* recombinase gene is inserted into the 3′ untranslated region of the *Pgr* gene in *Pgr^iresCre* mice (Yang et al., 2013), Cre expression represents the same expression kinetics as both PGR-B and PGR-A.

We observed β-gal expression in some theca/interstitial cells of the ovaries of *Pgr^iresCre* mice in this study, as both PGR-B and PGR-A are detectable by immunohistochemistry (Gava et al., 2004). Remarkable PGR expression is transiently induced by granulosa cells of the preovulatory follicle by stimulation of luteinizing hormone (LH) (Ismail et al., 2002). In the Soyal et al. (2005) study, strong β-gal staining was detected in the entire corpus luteum from *Pgr^Cre* with the reporter strain from 24 to 48 h after an injection of hCG (to mimic LH function). LH-induced PGR-expressing granulosa cells differentiate into luteal cells, which are negative for PGR (Soyal et al., 2005). We observed only spotted β-gal staining in the cross sections of the *Pgr^iresCre* corpus luteum even after hormonal treatment; this was probably due to a transient expression of PGR in the well-developed granulosa cells and IRES-mediated Cre expression.

It has been reported that genetic recombination by *Pgr^Cre* in the corpus luteum sometimes causes pregnancy failure in the conditional knockout mice. As shown by the use of LGR5, one of the well-established stem cell markers and a mediator of Wnt signal, the conditional knockout of *Lgr5* with *Pgr^Cre* results in subfertility due to the impairment of constitutive progesterone production from the corpus luteum (but not due to uterine functional defect) (Sun et al., 2014). Based on the β-gal staining pattern observed in this study, *Pgr^iresCre*

lines can be expected to be less effective for the study of ovarian function in conditional knockout mice.

Taking the present results together, we conclude that the *Pgr^iresCre* mouse strain is suitable for genetic modifications that target the female reproductive tract. *Pgr^iresCre* mouse effectively directed recombination of floxed gene in the endometrium as with *Pgr^Cre*. The benefit of using *Pgr^iresCre* lines is the maintenance of *Pgr^iresCre/iresCre* in a homogeneous manner. This may allow for more opportunities to obtain target genetic combinations and require less effort in genotyping. On the other hand, the following points should be paid attention to when using *Pgr^iresCre* lines: Cre activity in *Pgr^iresCre* induces genetic recombination in not only reproductive tissues but also brain tissues such as hypothalamus and hippocampus (Yang et al., 2013); rare unexpected germline recombination was found in *Pgr^iresCre* lines as reported in other Cre lines (Luo et al., 2020). The Cre-LoxP system is very effective for investigating gene functions in target tissues. Further research using *Pgr^iresCre* mice will contribute to reveal important information about the reproductive system and reproductive disorders in human and domestic animals.

CONFLICT OF INTEREST

Authors declare no conflict of interests for this article.

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