RU486-Inducible Recombination in the Salivary Glands of Lactoferrin Promoter-Driven Green Fluorescent Cre Transgenic Mice

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Summary: When compared with the many tamoxifen-activated Cre mouse lines available for gene manipulation studies, relatively few RU486-inducible Cre mice are in use, due to leakiness issues. Here, we report the generation of an RU486-inducible triple fusion gene (GCrePR1e), consisting of green fluorescent protein, Cre, and the progesterone receptor ligand-binding domain (F642-L901). We sought to improve the GCrePR1e by selecting a truncated human lactoferrin (Lf) promoter to drive its expression, based on the promoter's low basal activity and innate sensitivity to RU486. The resulting vector displayed decreased leakiness and increased Cre induction by RU486 through transcriptional and post-translational regulation in in vitro transfection assays. Inducible GCrePR1e expression was found in most organs of Lf-GCrePR1e transgenic mice and highly activated in the salivary gland, spleen, and lymph nodes. In the bigenic mouse generated by crossing the Lf-GCrePR1e mouse and the Cre reporter mouse (R26R-LacZ), we found that RU486-induced LacZ expression only in the mucous acini and striated ducts of the salivary gland and had very low background recombination in the untreated mice. Our results demonstrated that the Lf-CrePR1e vector was suitable for in vitro recombination in culture models, and Lf-CrePR1e transgenic mice could mediate spatially restricted and RU486-induced gene manipulation in the salivary gland. genesis 48:585–595, 2010.   © 2010 Wiley-Liss, Inc.

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INTRODUCTION

Conventional transgenic approaches lacking temporal and spatial control often suffer from drawbacks, such as early embryonic lethality that prevents the investigation of adult onset diseases, or complications resulting from phenotypes in unrelated organs that can mask the primary effect in target tissues (Metzger and Feil, 1999; Rajewsky *et al.*, 1996; Rossant and Nagy, 1995). To overcome these problems, temporal and spatial control of genetic manipulations can often be achieved by utilizing a DNA recombinase such as Cre (or Flp) and by flanking genes of interest or the target sequences with LoxP (or FRT) sites as a central framework for the conditional regulation of transgene expression (Sauer and Henderson, 1988; Sternberg and Hamilton, 1981). For instance, tissue-specific promoters can be used to restrict the spatial expression of Cre, while various means of inducing its activity can dictate when recombination occurs. The induction of Cre activity can be achieved both by increasing its expression using a drug-inducible promoter and by increasing the enzymatic ac-

tivity of the recombinase via conformational changes in the protein (Lewandoski, 2001; Utomo *et al.*, 1999). The latter approach is often achieved by fusing Cre to a truncated ligand-binding domain (LBD) of a steroid receptor that has been mutated so that it is highly sensitive to synthetic inducers but not to endogenous hormones. The fusion of Cre with the LBD of the estrogen receptor (ER) (Feil *et al.*, 1996), androgen receptor (AR) (Kaczmarczyk and Green, 2003), glucocorticoid receptor (GR) (Brocard *et al.*, 1998), and progesterone receptor (PR) (Kellendonk *et al.*, 1996) have all been reported. Fusions of Cre and mutated ERLBD, which are activated by the ER antagonists, tamoxifen or hydroxyl tamoxifen (4-OHT), have been widely used because of low leakiness (background recombination in the absence of inducers) and a high ratio of induction (Feil *et al.*, 2009). More than 20 different transgenic mouse lines with tissue specific promoter-driven expression of Cre-ERLBD have been generated (strains available from the Jackson Laboratory) (Feil *et al.*, 2009). CrePR1 (Cre fusion to 641-891 of PRLBD) was previously described (Kellendonk *et al.*, 1996), in which Cre activity was activated by nanomolar concentrations of RU486 (mifepristone), a PR antagonist. The mouse models based on CrePR1, with specificity in the brain (Kellendonk *et al.*, 1999b), heart (Minamino *et al.*, 2001), and skin (Berton *et al.*, 2000; Morris *et al.*, 2004) were established using this fusion and the results indicate that while administering RU486 induced gene deletion in targeted tissues, various degrees of recombination in the organs of the untreated mice (leakiness) were seen in all of the above models. By removing a cryptic slice donor at the 3′-end of Cre, a variant Cre*PR (650-914) exhibited reduced leakiness and enhanced sensitivity to RU486 (Wunderlich *et al.*, 2001). Transgenic mice with Cre*PR driven by the Krt1-15 promoter displayed RU486-induced-recombination in both the epidermis and the bulge (Ito *et al.*, 2007) while the leakiness associated with Cre*PR mice was not described.

Lactoferrin (Lf), a major iron-binding glycoprotein in milk, is expressed in many tissues (Teng, 2002; Teng *et al.*, 2002). Notably, it has been reported that RU486 induces Lf gene expression (Catalano *et al.*, 2003; Cheon *et al.*, 2002). Therefore, we assayed whether driving the expression of CrePRLBD with an RU486 inducible promoter, such as the Lf promoter, could further enhance Cre induction, through taking advantage of the transcriptional activation ability of RU486. In this study, we first developed a vector (GCrePR1e) expressing an EGFP-Cre-PRLBD (642-901) triple fusion protein under the CMV promoter. By tagging the recombinase with EGFP, the level and localization of GCrePR1e within the cells could be examined under a fluorescent microscope, which also helps to identify the site of potential recombination. To develop a dual activating Cre vector, the Cmv promoter was replaced by a truncated Lf promoter, which succeeded in reducing the leakiness and increasing the inducibility of Cre by RU486 in a cell

culture study. Next, we generated the Lf-GCrePR1e transgenic mouse and crossed it to the R26R-LacZ reporter mouse (Soriano, 1999) to analyze GCrePR1e expression and sensitivity to RU486 in vivo. The analysis of LacZ expression in the bigenic mice revealed that the DNA recombination induced by the RU486 was restricted to the salivary gland.

RESULTS

RU486-Inducible GFP-Cre and the Use of a Dual Fluorescence Reporter Vector to Report Cre Activity

To monitor and measure the Cre activity in cells, we developed two reporter vectors; the first, G-Luc, expresses firefly luciferase (Luc) as a result of Cre mediated recombination (Fig. 1a) and the second, G-Red, switches reporter expression from EGFP to DsRed upon recombination (Fig. S1). We created an expression construct containing a triple fusion gene, consisting of (N→C) EGFP, Cre, and PRLBD (F642-L901), driven by the Cmv promoter (Fig. 1a). The resultant triple fusion construct is referred to as GCrePR1e, because it contains PRLBD similar to PR1 (641-891 of PR) (Kellendonk *et al.*, 1996) with an extension at the C terminus. During construction of the vector, a linker with the amino acid sequence SSFE was introduced between Cre and PR1e, a region that may be important for conformational changes upon RU486 binding. To test whether Cre activity was induced by RU486, we transfected the GCrePR1e into CHO cells along with a G-Luc reporter vector and then applied RU486, progesterone, 17β-estradiol, or tamoxifen at various concentrations ranging from 1 nM to 100 nM. Luc activity was significantly increased by 2.3- to 3.6-fold after treatment with 100 nM RU486 (Fig. 1b). RU486 treatment resulted in higher Luc activity than did treatments with natural hormones. While there was also an increase in Luc activity of about 40–80% by 1 nM of progesterone, 17β-estradiol, and tamoxifen, this was not further increased using higher concentrations of hormones. In parallel, we transfected GCrePR1e along with the G-Red, followed by hormone or RU486 induction. The number of red fluorescent cells was increased by RU486 but not by progesterone, 17β-estradiol, or tamoxifen (Fig. S2). Although GCrePR1e catalyzed recombination was increased using a nanomolar concentration of RU486, residual recombination in the absence of RU486 mandated further improvement for reducing the basal Cre activity of GCrePR1e vector. To explore the source of this basal activity, we first tested whether hormones present in the serum normally added to culture media might result in Cre activation. We observed similar leakiness after replacing the serum with a replacement formula (serum replacement 1, Sigma) that does not contain growth factor, steroid hormones, or glucocorticoids (data not shown). Second, we tested whether the fusion of EGFP to the N
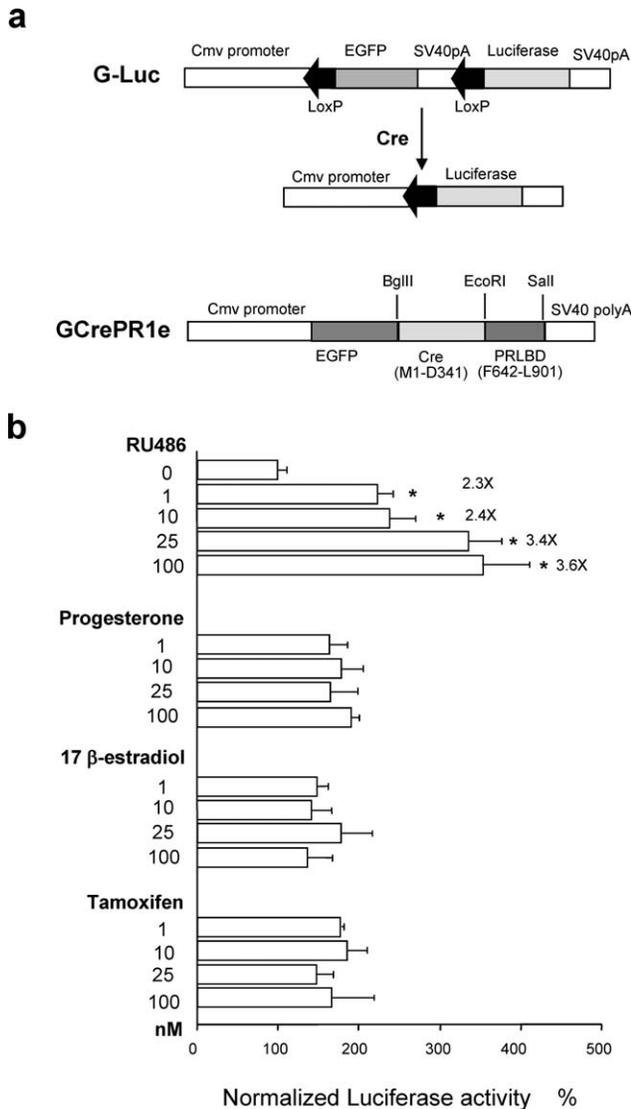
a

G-Luc

Cmv promoter   EGFP   SV40pA   Luciferase   SV40pA

LoxP   LoxP

Cre

Cmv promoter   Luciferase

GCrePR1e

Cmv promoter   BglII   EcoRI   SalI   SV40 polyA

EGFP   Cre   PRLBD
(M1-D341)   (F642-L901)

b

FIG. 1. (**a**) The structure of the G-Luc, and GCrePR1e vectors. In the presence of Cre, Luc expression is switched on as a result of recombination at the loxP sites. (**b**) CHO cells transfected by GCrePR1e and G-Luc vectors were either kept as untreated control cells or treated with 1, 10, 25, or 100 nM RU486, progesterone, 17β-estradiol, or Tamoxifen, followed by a luciferase activity assay. Normalized luciferase activity in ligand-untreated cells was designated as 100%. Error bars represent the mean + SEM, $n = 3$, *$P < 0.05$, when compared with untreated and different inducers using the same dose.

terminus of CrePR1e resulted in an unexpected GCrePR1e conformation that allowed the acquisition of Cre activity in the absence of ligand binding. We generated a CrePR1e construct that does not contain N-terminal EGFP and found that it had essentially the same activity as the GCrePR1e (Fig. S3). Thus, the source of basal activity could only be attributed to the region within CrePR1e. It has previously been suggested that cryptic splicing of Cre-PRLBD mRNA may

generate shorter transcripts that do not contain the LBD and could thus display basal and unregulated Cre activity. Replacing the [336]valine of CrePRLBD with alanine (V336A) significantly reduced the background activity of the recombinase and maintained its sensitivity to RU486 (Wunderlich *et al.*, 2001). Therefore, we performed RT-PCR to detect whether alternative splicing of GCrePR1e occurred, as has been suggested for other Cre-PRLBD constructs. We used a forward primer directed to the N terminus of Cre and a reverse primer directed to the SV40 PolyA sequence to detect any aberrant transcripts in GCrePR1e transfected CHO cells. However, we were not able to detect any PCR fragments other than the expected full-length transcripts (Fig. S4). In addition, we generated the same V336A mutation in our GCrePR1e construct and linked it with PRLBD starting at residue 650, in the hopes of similarly reducing the leakiness and improving RU486 induction (Wunderlich *et al.*, 2001). Our results indicated that the RU486-inducible recombinase activity of GCreV336APR was essentially nonexistent (data not shown).

For CrePRLBD fusions to efficiently recombine DNA in cells containing floxed sequences, they must localize to the nucleus. We therefore explored the translocation of GCrePR1e upon the addition of RU486 by tracking EGFP fluorescence in transfected cells. In untreated cells, GCrePR1e was more concentrated at the perinucleus but exhibited a broader distribution in the cytoplasm than GCreV336APR, which was completely restricted to the perinucleus (Fig. S5). Two hours after adding 1 nM RU486, GCrePR1e had begun to slowly relocate to the nucleus, and by 24 h, the majority of EGFP exhibited nuclear localization. In contrast, the majority of GCreV336APR remained outside the nucleus 24 h after adding RU486, which may explain the failure of GCreV336APR in mediating recombination. Without fusing with any LBD, GCre was primarily inside the nucleus (Fig. S5).

Combining RU486-Induced Activity and Transcription to Create a New RU486-Inducible Cre Vector

By using a dual GLVP/CrePR system that simultaneously activates CrePR at both transcriptional and post-translational levels, Kyrkanides *et al.* demonstrated decreased leakiness and increased recombinase induction in a cell culture system (Kyrkanides *et al.*, 2003). We searched for similar approaches using RU486 to simultaneously induce the expression of Cre and its catalytic activity. We searched for genes that were reported to have their expression increased by RU486 and identified human Lf as a candidate gene (Catalano *et al.*, 2003; Cheon *et al.*, 2002). To determine the length of the Lf promoter required for RU486-induced expression, various lengths of the human Lf promoter, designated as LfA (−1008 to −24), LfB (−457 to −24), and LfC (−344 to −24), were cloned into the
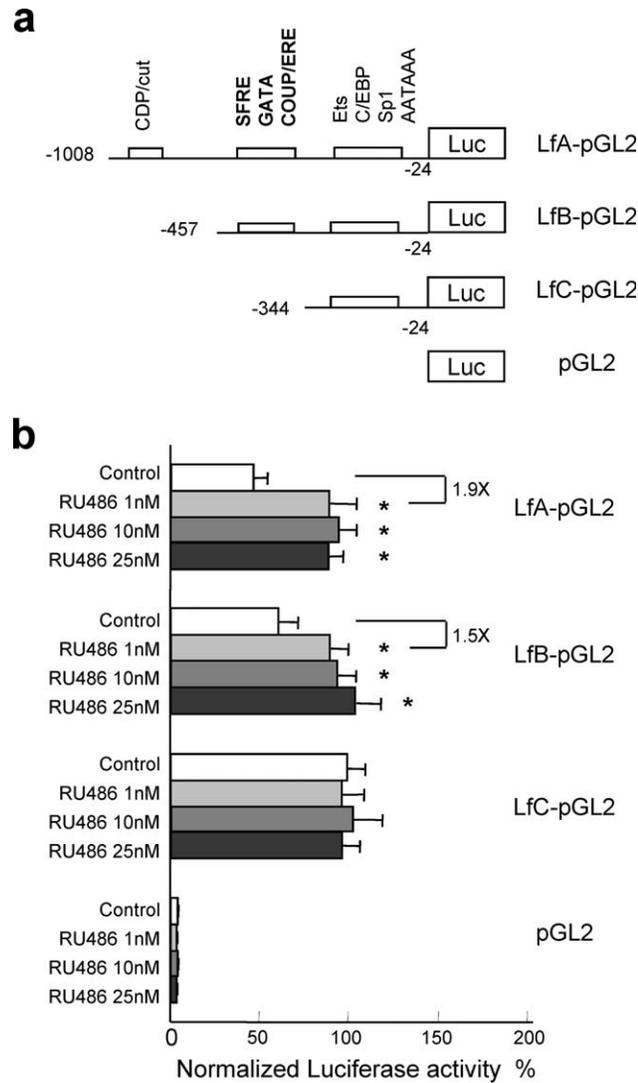
a

b

a

b

c

FIG. 2. (**a**) The truncated Lf promoter-Luc reporter constructs: LfA(-1008 to -24), LfB (-457 to -24), and LfC (-344 to -24) linked to the pGL2 basic vector. Potential transcription factor binding elements in this putative promoter region are: CCAAT displacement protein (CDP/cut, -850 to -820), SF-1 binding element (SFRE), GATA, estrogen response element overlapping with a chicken ovalbumin upstream promoter (ERE/COUP) from -409 to -349, and an immediate early region which contains two Sp1 sites, ETS, Myb, and C/EBP binding elements (-85 to -35). (**b**) The Lf reporter activation by RU486 in CHO cells. The luciferase activity in CHO transfected by LfC-pGL2 without treatment was designated as 100%. *$P < 0.05$, when compared to its own untreated control. Error bars represent the mean $+$ SEM, $n = 3$.

FIG. 3. (**a**) The structure of LfA-, LfB-, LfC-, and GCrePR1e vectors. (**b**) The induction of Cre activity by RU486 in G-Luc MB231 reporter cells after transfection of LfA-, LfB- (black bars), or LfC-CrePR1e vector. Luc activity in the 10 nM RU486 treated LfA-GCrePR1e group was designated as 100%. (**c**) The comparison of LfB-GCrePR1e (black bars) and Cmv-GCrePR1e regarding leakiness and RU486 induction in G-Luc MB231 reporter cells. The luciferase activity in the 10 nM RU486-treated Cmv-GCrePR1e group was designated as 100%. The data shown were from a representative study with triplicate samples and the studies were repeated at least twice. Error bars represent the mean $+$ SEM, $n = 3$.

pGL2 basic vector (Promega) and these reporter vectors were transfected into CHO cells, followed by RU486 treatment (Fig. 2a). The shortest construct, LfC-pGL2, which contains putative binding sites for transcription factors Ets, C/EBP, Sp1, and a TATA-like sequence, resulted in high basal activity but was not induced by RU486 (Fig. 2b). A further upstream region that contained binding sites for SFRE, GATA, and
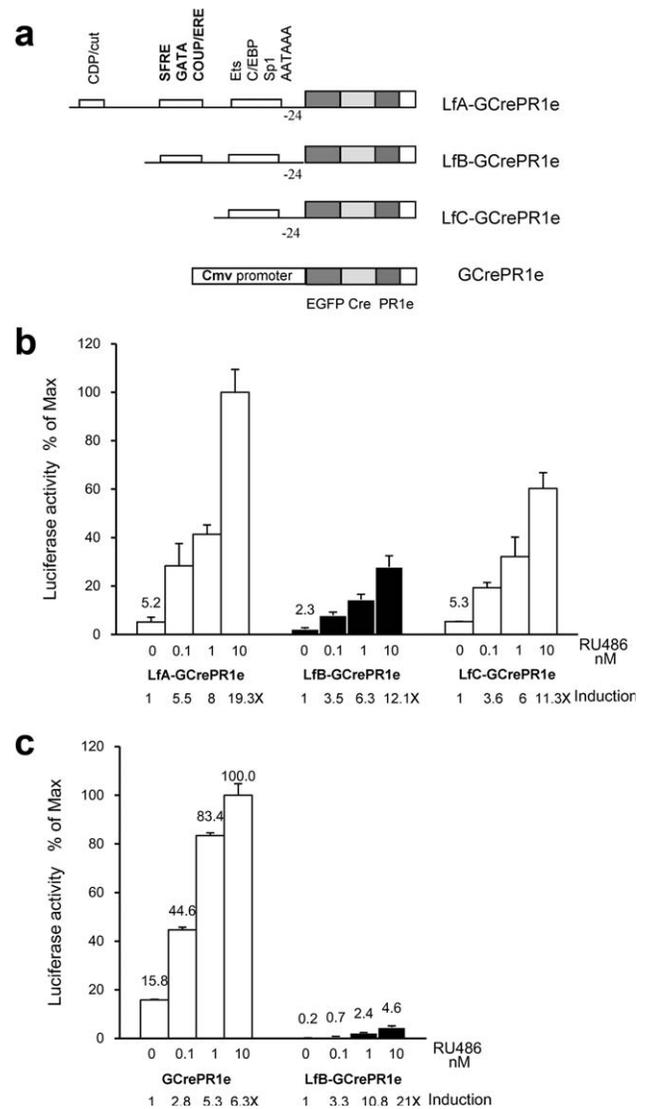
COUP/ERE, functions as a suppressive cassette; the constructs LfA-pGL2 and LfB-pGL2, both contained this region, displayed reduced basal activity and had a 1.5- to 2-fold increase of reporter expression using RU486. We therefore replaced the Cmv promoter in GCrePR1e with the Lf promoter fragments to test basal levels and the induction of Cre function (Fig. 3a). One drawback to the cotransfection assays (shown in Fig. 1) is that
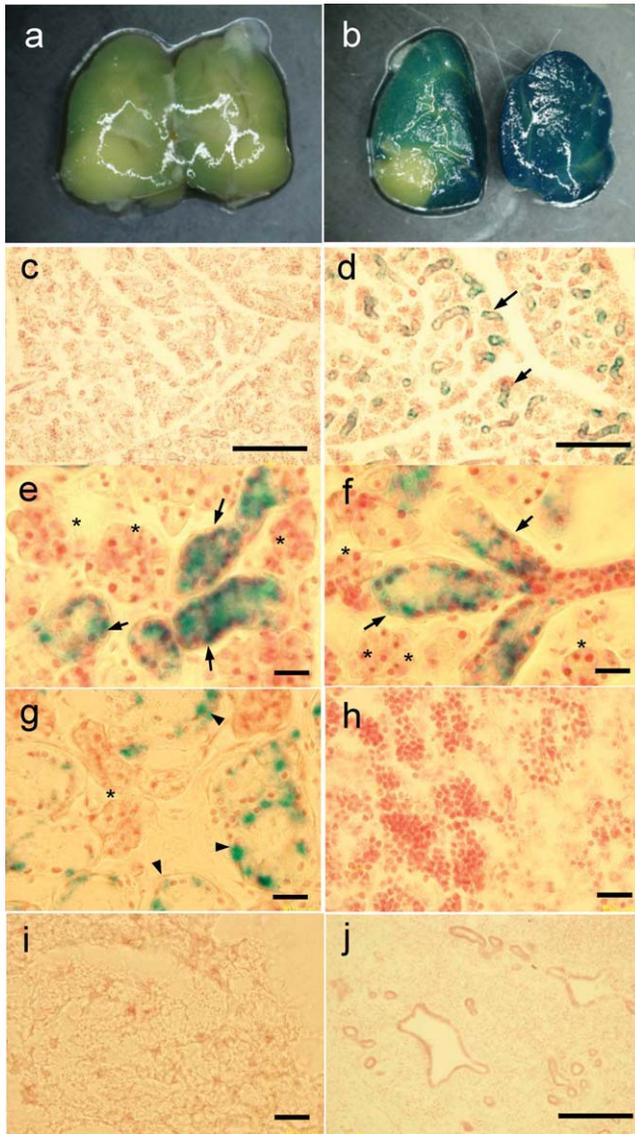
FIG. 4. X-gal staining of tissues from LfB-GCrePR1e (line #15):: R26R-LacZ bigenic mice showing induction of recombinase activity by implanting one RU486 pellet for 21 days. Whole mount staining of the submandibular gland from the bigenic mouse treated by placebo (**a**) had strong LacZ induction using RU486 treatment (**b**). X-gal staining of cryosections (**c–g**) confirmed that very rare recombination occurred in placebo-treated salivary glands (c) and RU486 treatment (d–g) induced recombination in almost all striated ducts (arrows) and mucous acini (arrow heads). Recombination was completely absent in serous acini (asterisks in e–g**)**. Following RU486 treatment, recombination was also absent in the spleen (**h**), mammary gland (**i**), and uterus (**j**). Scale bar = 200 μm (c, d, j) or 20 μm (e–i). The same analyses were performed using R26R-LacC::Lf-GCrePR1e line #7 and line #27 (Fig. S9).

an increase in Luc activity or the appearance of red fluorescent cells (Fig. S2) may result from the recombination of the reporter vector in the cytoplasm, as opposed to reflecting the recombination of chromosomal DNA in the nucleus. To accurately estimate recombination in chromosomal DNA following Cre

induction, we generated G-Luc stably transfected MDA-MB231 cells and used them as a model for the nuclear DNA recombination study (Chen *et al.*, 2009). We transfected LfA-GCrePR1e, LfB-GCrePR1e, or LfC-CrePR1e into the G-Luc reporter cell line and measured Luc activity induced by RU486 addition (Fig. 3b). All three vectors displayed a dose-dependent increase of Luc activity in response to RU486, and administration of 10 nM RU486 increased Luc activity more than 10-fold. LfA-GCrePR1e appeared to have the highest reporter induction in response to RU486 but also exhibited higher background, as did that of LfC-GCrePR1e. Because LfB-GCrePR1e exhibited the lowest basal recombinase activity and yet remained highly inducible by RU486, we considered it the most suitable candidate for subsequent animal study among the three vectors. We compared LfB-GCrePR1e to Cmv-GCrePR1e in the G-Luc reporter cell line (Fig. 3c) and found that LfB-GCrePR1e reduced the background activity roughly 80-fold (from 15.8% to 0.2%) and increased the induction from roughly 6-fold (of Cmv-GCrePR1e) to 21-fold. We also transfected the LfB-GCrePR1e and G-Red vectors in CHO cells and compared the recombination induced by RU486 with that of progesterone, 17 β-estradiol, and tamoxifen (100 nM). The results confirmed the RU486 dose-dependent increase of red fluorescent cells, and showed that the recombinase activity of LfB-GCrePR1e was not induced by at least two orders of magnitude more concentrated progesterone, 17 β-estradiol, or tamoxifen (Fig. S6). To confirm that RU486 induces the transcription of a LfB-driven vector, we measured the GCrePR1e expression in response to the added RU486 using quantitative PCR in a cell culture model. The results indicated that RU486 led to a transient increase of GCrePR1e transcripts up to three folds (Fig. S7).

Lf-GCrePR1e Mice Display RU486-Inducible Recombination in the Salivary Gland

As in vivo function is the ultimate test of an RU486-inducible Cre vector, we generated transgenic mice using the LfB-GCrePR1e vector (thereafter termed the Lf-GCrePR1e mouse), crossed them to the R26R-LacZ reporter mice, and tested RU486-inducible recombination in the resultant bigenic mice. We have performed studies using mice originating from four (line #7, #11, #15, and #27) out of twelve Lf-GCrePR1e mouse founders, while most studies were carried out on line #15. The Southern blots of these mice lines are shown in Figure S8. To induce Cre activity, a slow-release RU486 pellet (21-days release, 2 mg/pellet) was implanted into the bigenic mice and the expression of LacZ in mice treated by the placebo or RU486 was examined by β-gal staining of whole mount tissues or cryosections. As shown in Figure 4, only the salivary glands of the RU486 treated mice displayed X-gal staining (Fig. 4b) while salivary glands of the placebo treated mice did not display staining (Fig. 4a,c). Recombination was restricted to the mucous aci-
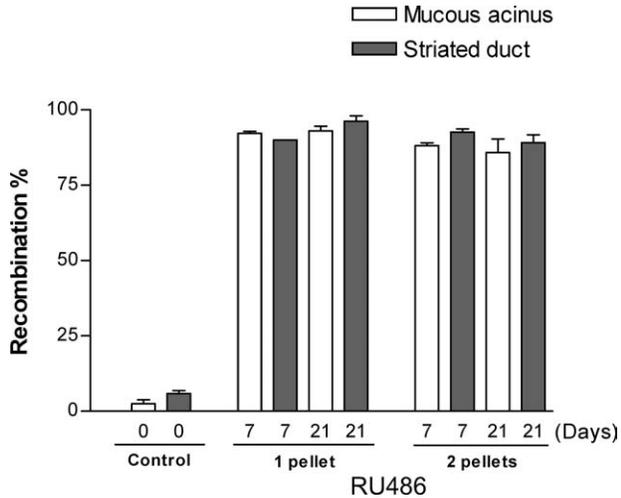
FIG. 5. The efficiency of recombination induced by RU486 in the salivary gland. Mice from transgenic line #15 were implanted with placebo, one, or two RU486 pellets, and the salivary glands were harvested after seven or twenty-one days. Efficiency was determined by the percentage of blue mucous acini or striated ducts contained in each X-gal stained tissue section. Error bars represent the mean + SEM, $n = 3$.

nous and striated ductal cells but not to the serous acinous cells between the buds (Fig. 4d, e, f, g). X-gal staining was not present in other tissues with high Lf expression, such as the spleen, mammary glands, and uterus of RU486-treated mice (Fig. 4h, i, j). To explore the effect of RU486 dosing and induction time on recombination efficiency, we implanted one or two RU486 pellets in the mice and harvested the salivary gland after seven or 21 days. By counting the percentage of blue mucous acini or striated ductal cells contained in each section, we found that after seven days the floxed cassette was deleted in ∼90% of these cells with LacZ expression. Implanting an additional RU486 pellet or induction for 21 days led to similar results (Fig. 5). We measured the spontaneous recombination in these cells of placebo-implanted bigenic mice and found it to be at very low frequency, whereas 3–5% of mucous acini or striated ducts contained one or more blue cells (Fig. 5). In addition to line #15, we found similar low background Cre activity and RU486 recombination restricted to the salivary gland in two other mouse lines (Fig. S9).

To study the induction of GCrePR1e transcription by RU486 in vivo, we measured the expression of GCrePR1e transcripts by quantitative PCR in various tissues and performed immunostaining on tissue sections from placebo or RU486-treated mice using a GFP antibody to stain GCrePR1e and a Gr-1 antibody to stain the neutrophils. As shown in Figure 6a, the basal expression of GCrePR1e was higher in the salivary gland, the spleen, the uterus, and the lymph node than in other tissues. RU486 appeared to increase GCrePR1e expression in multiple tissues, but the increase was not significant. Similar expression patterns were found in two other

transgenic lines #7 and #27 (data not shown). RU486 induces the expression of GCrePR1e in cells lining the striated ducts of the salivary gland (Fig. 6b), in agreement with the LacZ expression results. In the spleen and lymph nodes, the cells expressing GCrePR1e are identified as neutrophils by Gr-1 and GFP staining colocalization. GCrePR1e was only weakly detected by immunostaining in the mammary gland or the uterus after RU486 treatment. Finally, we studied if Lf expression in the salivary gland and the spleen was induced by RU486, an indication that transgenic LfB promoter activity could mimic the expression of the murine endogenous Lf gene. As shown in Figure 7, RU486 significantly increased Lf expression in the salivary gland, primarily in the striated ductal cells. By using Lf and Gr-1 antibodies, we also found many double labeled neutrophils in both the non-induced and the induced spleen, in agreement with the finding that neutrophils highly express Lf. Therefore, our results demonstrate that the Lf-GCrePR1e mouse lines exhibit a salivary gland specific RU486-induction of fluorescent Cre with very low leakiness.

DISCUSSION

The purpose of this study was to develop a vector that contained an inducible green fluorescent Cre that could be dually activated both transcriptionally and enzymatically by RU486 for conditional transgenic research. We initially selected a truncated Lf promoter to drive the GCrePR1e expression based on its innate sensitivity to RU486 and on the assumption that Lf gene is expressed in many organs; resultant Lf-GCrePR1e mice could therefore achieve an RU486-inducible deletion in multiple organs. However, our data showed that in response to RU486, Lf-GCrePR1e mice deleted the floxed gene only in the salivary gland but not in other tissues that normally express high levels of Lf. Nevertheless, the pattern of gene deletion, although restricted to the salivary gland, was consistent with the known expression of Lf in this organ.

Reporter constructs that switch on or off the expression of the reporter (including GFP, RFP, Luc, CAT, β-gal) as the indicator for Cre mediated recombination have been previously reported (Kaczmarczyk and Green, 2001; Kellendonk *et al.*, 1996; Yang and Hughes, 2001). The G-Red reporter used in this study allows direct in vitro or in vivo imaging for the appearance of red fluorescence resulting from recombination without the need for substrates and enzymatic reactions, while the G-Luc vector and stably expressing cell line enable quantitative measurements of recombination using the standard Luc assay. Studies using cotransfection of reporter constructs and Cre containing vectors are not able to differentiate between recombination occurring in the cytoplasm and that affecting genomic DNA; as a result, Cre activity leakiness may be overestimated and RU486 induction simultaneously underestimated by recombination in the cytoplasm. By using a G-Luc stable cell line, these issues were circumvented and the recombination of genomic
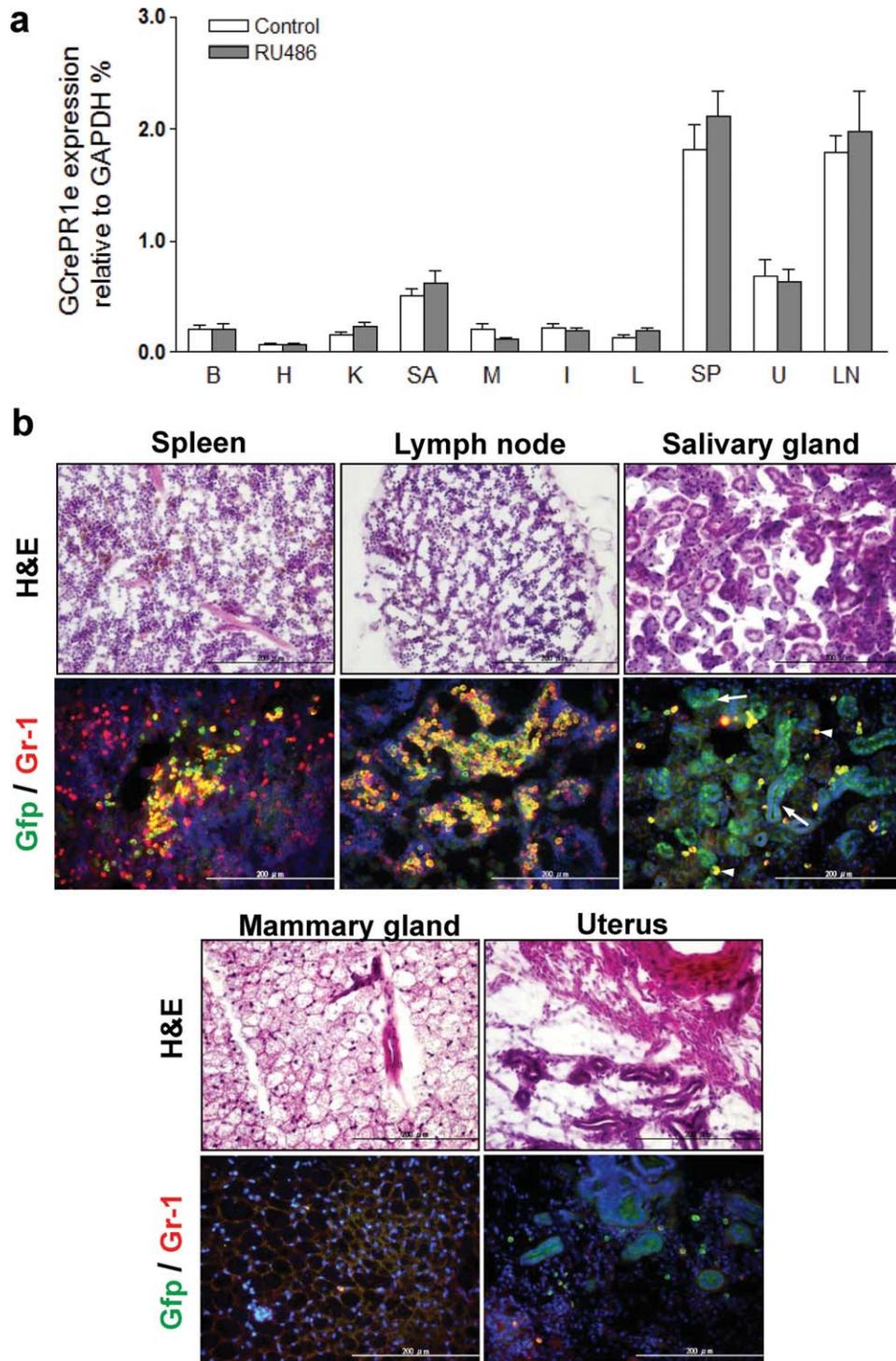
FIG. 6. The expression of GCrePR1e in Lf-GCrePR1e mice. (**a**) GCrePR1e expression in tissues isolated from mice (line #15) that were placebo or RU486-treated for five days. RNA was treated as in Figure S4 and S7 to eliminate genomic DNA contamination. Higher GCrePR1e expression was found in the spleen, lymph nodes, uterine, and salivary gland. Similar expression pattern was found in two other transgenic lines #7 and #27 (data not shown). RU486 appeared to increase GCrePR1e expression in multiple tissues, but the increase was not significant. $n = 4$. B, brain; H, heart; K, kidney; SA, salivary gland; M, skeletal muscle; I, intestine; L, lung; SP, spleen; U, uterus; LN, lymph node. (**b**) The detection of GCrePR1e proteins in RU486 treated Lf-GCrePR1e mice (line #15). The staining used was H&E, GFP (green), Gr-1 (red), and DAPI (blue). GCrePR1e-expressing cells in the spleen and the lymph node were also positive for Gr-1. Not all Gr-1 positive cells expressed GCrePR1e. The primary locations for GCrePR1e expression in the salivary gland were at the striated ductal cells (arrows) and neutrophils (arrow heads). Scale bar = 200 μm.
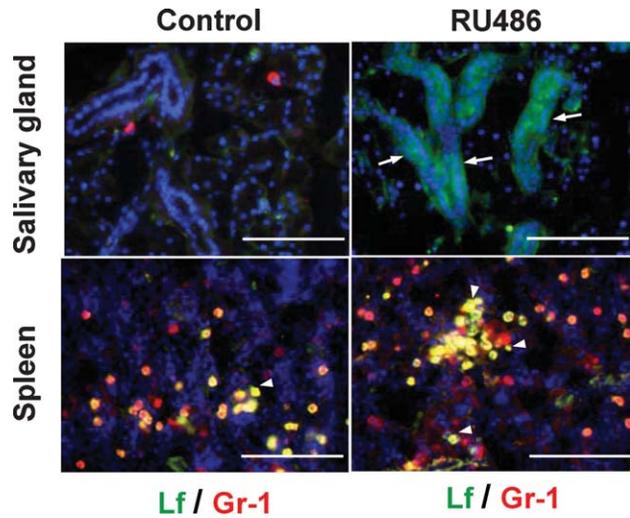
Control   **RU486**

FIG. 7. The expression of Lf in tissues of control and RU486-treated mice (line #15). The staining used was H&E, Lf (green), Gr-1 (red), and DAPI (blue). Striated ductal cells (arrows) in the salivary gland and neutrophils (arrow heads) in the spleen demonstrated induced Lf expression in RU486-teated mice. Scale bar = 100 μm.

DNA was more accurately measured. In studies that counted the ratio of recombined cells to the total number of cells, Cre-PRLBD fusions were reported to achieve 10 to 100+-fold increases in Cre activity upon the addition of RU486 (Kellendonk *et al.*, 1999a; Kellendonk *et al.*, 1996; Wunderlich *et al.*, 2001). However, in the report of the dual GLVP/CrePR system (Kyrkanides *et al.*, 2003) that theoretically possesses the lowest background and the highest induction by RU486, a reporter cell line that measures Luc activity only in recombined cells was used to measure Cre activity, and the maximum induction by RU486 was found to be only 20-fold. In this study we used a similar G-Luc cell line and found the maximum LfB-GCrePR1e induction by RU486 to be also approximately 20-fold.

Phage Cre contains a nuclear localization sequence (NLS) between residues R100 and V230 that allows Cre to efficiently enter the nucleus (Le *et al.*, 1999). Indeed, localization of the GFP-Cre fusion was primarily in the nucleus (Fig. S5). Several NLSs have been identified in the PR and the region with the most dominant nuclear targeting effect was identified between residues 638–642 (Guiochon-Mantel *et al.*, 1989; Tyagi *et al.*, 1998; Ylikomi *et al.*, 1992) of the receptor. Another ligand binding-induced NLS was identified as the helices 1-7 between residues 680 and 810 of the PRLBD (Wan *et al.*, 2001). Our results show that fusion of the PRLBD to the C-terminus of EGFP-Cre disables the endogenous nuclear targeting determinants in Cre, resulting in the resting distribution of GCrePR1e in the cytoplasm and that nuclear translocation is induced following the adding of RU486 (Fig. S5). The underlying mechanism of GCrePR1e in disabling the nuclear targeting feature of Cre by fusion with PRLBD is still unclear. While main-

taining the level of GCreV336APR expression in cells (as demonstrated by the green fluorescence in Fig. S5), the removal of the SSFE linker and PR residues 642-649, along with the conservative V336A mutation, abolished both the Cre activity (data not shown) and ligand-induced nuclear translocation (Fig. S5). One possible explanation is that removing these sequences can result in Cre being put into a conformation that results in blockage of its catalytic action, regardless of ligand binding. The SSFE linker is encoded by the *SacI-HindIII-EcoRI* sequence within the pEGFP vector, which is not likely to play a significant role in determining PRLBD function. Therefore, PR residues 642-649 must be critical to the ligand-induced translocation and activity of CrePR. RU486-induced GCrePR1e nuclear translocation is instrumental for applying this tool in genomic manipulation. To our knowledge, this is the first report demonstrating the nuclear translocation of Cre-PRLBD triggered by RU486. Inclusion of EGFP in the triple fusion has greatly facilitated observation of the GCrePR1e translocation.

Imperfect palindromic EREs and SFREs in the human Lf promoter mediate the Lf gene activation in responding to natural and synthetic estrogen (Teng, 2002). We found this region to be a strong suppressive element for basal Lf promoter activity (Fig. 2), in agreement with previous reports (Liu and Teng, 1992), and also mediates RU486 induction of the Lf promoter. How RU486, a PR antagonist, activates the Lf promoter through ERE and SFRE remains unclear. A possible explanation is that RU486 can behave like an ER agonist and activates ERα, which results in increased ERα binding to ERE and SFRE (Jeng *et al.*, 1993). Gene specific interplays of factors, including the ratio of ER α/β isoforms, interactions between particular ERE/SFRE sequences, neighboring transcriptional factor binding elements and transcription factors, and multiple nuclear receptors, are also likely involved. Examples of these mechanisms have been demonstrated to act on other ERE containing gene promoters (Hall *et al.*, 2001, 2002; Hall and Korach, 2002).

The RU486-induced gene deletion was restricted to the mucous acini and striated ductal cells of the salivary gland but was not found in other tissues, such as neutrophils in the spleen and the lymph node, the uterus and the mammary gland that are known to express Lf. Because a truncated human Lf promoter was used to drive GCrePR1e expression, what we observed on GCrePR1e may not completely recapitulate the expression pattern and the response of the Lf gene in mice. Although we have shown that RU486 caused a transient increase of GCrePR1e transcripts in the cell culture model (Fig. S7) and that the level of Lf expression in neutrophils and salivary gland indeed increased following RU486 administration, we were not able to demonstrate a similar increase of GCrePR1e expression in these tissues. It is likely due to the timing of harvesting tissues following RU486 treatment and to a small induction by RU486. In addition, we did not detect any recombined ROSA alleles by PCR (data not shown) or LacZ expres-

sion in the uterus, spleen, or the lymph node, although GCrePR1e in these tissues was expressed at the level comparable with that in salivary gland of untreated or RU486-treated mice (see Fig. 6). The reason for the lack of recombination in uterus is unclear; however, it is likely due to the life of neutrophils being too short for LacZ expression to be seen in the spleen or in the lymph nodes. In contrast to lines such as the MMTVLTR-Cre mouse (Wagner *et al.*, 1997) that achieved gene deletion in the salivary gland that was often found accompanied by recombination in other secretory tissues, Lf-GCrePR1e mice display both specificity and high sensitivity to RU486 induction (achieved high recombination efficiency in seven days).

In summary, we have engineered a green fluorescent RU486 inducible Cre recombinase construct driven by the Lf promoter. The resultant construct has reduced the common leakiness associated with the Cre-PRLBD approach and is strongly induced by low doses of RU486. Lf-GCrePR1e mice display efficient RU486-inducible DNA recombination in the salivary glands and promise to become a useful genetic tool.

MATERIALS AND METHODS

All cell culture medium reagents and chemicals used in this study were from Invitrogen or Sigma-Aldrich, respectively, or were otherwise indicated.

Vector Construction

Two Cre reporter vectors were used in this study: Cmv-LoxP-EGFP-LoxP-DsRed (G-Red) and Cmv-LoxP-EGFP-LoxP-Luc (G-Luc). Full-length EGFP coding sequence with stop codon and three SV40 PolyA sequences was modified in the pEGFP-C2 vector (Clontech) and inserted between the two LoxP sites of the puc1015LoxGFPLox vector (kindly provided by Chu Chen, UCSD). The LoxP cassette containing EGFP was inserted into the pDsRed2-N1 vector (Clontech) to create the G-Red reporter. The construction of G-Luc vector was performed as described previously (Chen *et al.*, 2009). Cre DNA recombinase coding sequence was amplified from the pGIKS-Cre vector obtained from ATCC (#87490) with the forward primer *BglII-SacI*-Cre (CAGATCTCGAGCTCAATGTCCAATTTACTGA) and the reverse primer Cre-*HindIII-EcoRI* (GAATTCGAAGCTT GAATCTTCCAGCAGGCGCACCA). The resulting 1040bp PCR fragment was cloned into the *BglII/EcoRI* sites of the pEGFP-C2 vector to obtain the EGFP-Cre (GCre) vector. Human progesterone receptor hormone binding domain (F642 to L901 of PRLBD) was amplified from cDNA isolated from human ZR75-1 breast cancer cells with the forward primer *EcoRI*-hPRLBD (GAATTCAATAAAGTCA GAGTTGTGAG) and the reverse primer hPRLBD-*SalI* (CGTCGACTGGATAAATGTATTCAAGCAGTACAG). The resulting PRLBD fragment was inserted in frame with the C terminus of GCre to created GCrePR1e. Human Lf promoter was isolated from genomic DNA of ZR75-1 cells by PCR using the following primers: LfA sense (CCAATGTGTCCCACAGGCA), LfB sense (TGCTGAGC CAAGGTGAAAGC), LfC sense (GACCCTCTGCTCTG TGT) and antisense (CAAACGAAGGCTCTGCCACTT) starting from the positions −1004, −457, −344, and −24 relative to the ATG of human Lf gene. The resultant PCR products were inserted into GCrePR1e in the place of the Cmv promoter to generate the LfA-GCrePR1e, LfB-GCrePR1e, and LfC-GCrePR1e vectors. Similarly, the LfA-pGL2, LfB-pGL2, and LfC-pGL2 luciferase reporters were created by inserting the corresponding fragments into pGL2 vector (Promega). All vectors were verified by DNA sequencing reactions.

Cell Culture and Transfection

Chinese hamster ovary cancer cells (CHO-K1) were maintained in F-12 medium supplemented with 10% fetal bovine serum and the antibiotics penicillin, streptomycin, and amophotericin B (PSA) in a 95% air and 5% CO_2 humidified incubator. Human mammary carcinoma MDA-MB231 (MB231) cells (ATCC#HTB-26) were maintained in RPMI 1,640 medium with 10% FBS and PSA. Prior to seeding cells for transfection using the PolyFect (Qiagen) reagent, the cell numbers were counted to ensure that an equal number of cells were used in each experiment. The ratio of reporter vector to Cre vector used in transfection was three to one wherever applicable.

Cre Induction and the Luciferase Activity Assay

CHO cells after overnight transfection were treated with RU486, progesterone (P4), 17 β-estradiol (E2), and tamoxifen at the indicated concentrations in culture medium to induce Cre activity. After incubation for 24 h, fluorescent microscopic images were taken or the Luc reporter assays were performed by using a Luc assay kit (Promega) as appropriate for the experiments. A RSV β-galactosidase control vector was cotransfected into cells and followed by β-gal assay (Promega). The luciferase activity readings were normalized to the protein quantity or to the β-gal activity in cell lysates.

Fluorescent Microscopy and Image Analysis

The expression of EGFP and DsRed in cells was observed using Olympus IX71 fluorescence microscope. Immunofluorescence staining was performed on 6 μm frozen sections of 4% paraformaldehyde-perfused organs. Rabbit anti-GFP (Abcam) or anti-Lf antibody (Upstate) were used with Alexa Fluor 488 goat anti-rabbit IgG. Rat anti-mouse Ly-6G (Gr-1) antibody (eBioscience) was used with Northern Light 557 anti-rat IgG. DAPI contained in antifade reagent was used to stain the nucleus.

Animal Studies

All animal experiments were conducted in accordance with accepted standards of animal care and were approved by the Institutional Animal Care and Use Committee of the National Health Research Institutes, Tai-

wan. FVB/NarL transgenic mice were generated by the Level Inc. (Taipei, Taiwan) using the ApaL1 and DraIII digested LfB-GCrePR1e vector. A total of twelve transgenic founders were generated and transgenic progeny from four of the founders (#7, #11, #15, and #27) were crossed with R26R-LacZ mice and used in this study. Genotyping of bigenic mice was performed by PCR using the primers CATGGTCCTGCTGGAGTTCGTG, GAATTCGAAGCTTGAATCTTCCAGCAGGCGCACCA, and primers to identify R26R-LacZ positive mice (Soriano, 1999). An RU486 slow releasing pellet (total dosage of 2 mg per pellet, Innovative Research of America) was subcutaneously implanted, producing a constant daily dose of 3 mg/Kg body weight for 21 days. The placebo pellets (Innovative Research of America) were similarly implanted in the control mice. Toxicity from RU486 was not seen. Seven or twenty-one days after implanting the pellet, these mice were sacrificed followed by whole body perfusion with 4% paraformaldehyde. LacZ tissue staining was performed on 20-μm cryosections according to a previously described protocol (Lobe *et al.*, 1999). After staining, the sections were counter stained using Fast Red and mounted for histology examination. Efficiency of recombination was determined by the percentage of blue mucous acini or striated ducts contained in each X-gal stained tissue section. Three mice were used for each group and at least six sections were counted for each mouse.

ACKNOWLEDGMENTS

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