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A CK19^{CreERT} Knockin Mouse Line Allows for Conditional DNA Recombination in Epithelial Cells in Multiple Endodermal Organs

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Summary

Cre/LoxP-mediated DNA recombination allows for gene function and cell lineage analyses during embryonic development and tissue regeneration. Here, we describe the derivation of a *K19^{CreERT}* mouse line in which the tamoxifen-activable CreER^T was knocked into the endogenous cytokeratin 19 locus. In the absence of tamoxifen, leaky Cre activity could be detected only in less than 1% of stomach and intestinal epithelial cells, but not in pancreatic or hepatic epithelial tissues. Tamoxifen administration in postnatal animals induced widespread DNA recombination in epithelial cells of pancreatic ducts, hepatic ducts, stomach, and intestine in a dose-dependent manner. Significantly, we found that Cre activity could be induced in the putative gut stem/progenitor cells that sustained long-term gut epithelial expression of a Cre reporter. This mouse line should therefore provide a valuable reagent for manipulating gene activity and for cell lineage marking in multiorgans during normal tissue homeostasis and regeneration.

Keywords

lineage tracing; pancreas; small intestine; colon; liver; kidney; stomach; Cre

The Cre/LoxP-based technology allows for functional analyses of essential genes in specific organs by gene inactivation or controlled ectopic gene expression (Branda and Dymecki, 2004; Lewandoski, 2001; Sauer and Henderson, 1988). When combined with detectable marker protein expression, Cre-LoxP allows for cell lineage analyses in living animals (Branda and Dymecki, 2004; Gu *et al.*, 2003). Upon modifying Cre to produce a tamoxifen (TM)-dependent molecule, CreER^T, it is now possible to control Cre activity both spatially and temporally (Metzger and Chambon, 2001). This feature allows for dissecting the genetic requirements for cell/tissue homeostasis and for following cell lineages during tissue regeneration.

We have derived a *K19^{CreERT}* knockin allele to recombine DNA in epithelial cells of several adult organs. *K19* encodes an intermediate filament protein (Moll *et al.*, 1982) that is expressed in multiple cell types from the epiblast stage and is maintained in multiple epithelial cell types of later embryonic and postnatal stages (Bosch *et al.*, 1988; Lane *et al.*, 1983; Moll *et al.*, 1982; Quinlan *et al.*, 1985). For example, *K19* is highly expressed in the pancreatic ducts of the adult pancreas (Deramaudt *et al.*, 2006), but is absent or weak in acini and islets (Brembeck *et al.*, 2001). Similarly, *K19* is highly expressed in the liver duct

cells but not in the mature hepatocytes (Nishikawa *et al.*, 1996). Because studies have suggested that the pancreatic duct and liver duct cells may behave as progenitor or stem cells for tissue regeneration (Dorrell and Grompe, 2005; Hu *et al.*, 2007; Xu *et al.*, 2006), *K19^{CreERT}*-based cell lineage studies could reveal the potential fate of these ductal cells during regeneration and tissue homeostasis. Additionally, *K19^{CreERT}* could allow for temporally controlled gene inactivation and/or activation in epithelial cells to analyze gene function during cell renewal and tissue maintenance, a tribute that cannot be addressed with the existing *K19^{Cre}* mouse line that is active in progenitors for all tissues of the embryo proper (Means *et al.*, 2005).

We derived a *K19^{CreERT}* allele by replacing *K19* ATG with a CreER^T-cDNA followed by a SV40 polyadenylation signal (see Fig. 1). This design minimally altered *K19* transcription regulatory elements while producing a CreER^T message with a short 3'-UTR. Inclusion of a polyadenylation signal 3' to the CreER^T sequence prevented the transcription of the five noncoding exons of the endogenous *K19* gene. Otherwise, the presence of these noncoding exons in CreER^T mRNA could trigger nonsense-mediated mRNA degradation (Conti and Izaurralde, 2005; Doma and Parker, 2007). Thus, adding an extra polyadenylation signal immediately down-stream of CreER^T cDNA is designed to enhance CreER^T production. We expected that *K19^{CreERT}* would produce CreER^T, which could be activated by application of TM, in most, if not all *K19*-expressing cells.

R26R^{EYFP} reporter mouse was utilized to monitor Cre activity by virtue of EYFP expression that is dependent upon Cre-mediated recombination (Srinivas *et al.*, 2001). Because EYFP is a small protein, it readily diffuses between the cytoplasm and the nucleus but often appears more abundant in the nucleus. TM was administered to *K19^{CreERT}⁺; R26R^{EYFP}⁺* animals of different ages, at P0 (Day 1 after birth), P3, or P6 with 0.2–0.5 mg TM or at 8 weeks of age with (4 mg × 3 doses) TM. Four days after the last TM injection, EYFP expression in several endodermally derived organs was monitored.

We first examined EYFP expression in *K19^{CreERT}⁺; R26R^{EYFP}⁺* pancreas, which contains the exocrine acini, pancreatic ducts, and endocrine islet cells, with TM administration at several stages (Slack, 1995). Immunohistochemical assay (IHC, Fig. 2a–d) or direct EYFP fluorescence observation (FL, Fig. 2e) detected no pancreatic cells expressing EYFP without TM administration (Fig. 2a and data not shown). Yet TM administration to adult (Fig. 2b–d) or neonatal (Fig. 2e) *K19^{CreERT}⁺; R26R^{EYFP}⁺* animals induced widespread EYFP expression in the pancreatic ducts 1 week after TM administration. In animals that received 0.5 or 12 mg of TM at P0 or 8 weeks of age, respectively, from 10–45% of interand intralobular duct cells ($n = 4$ for P0 and $n = 6$ for adults) produced EYFP. No EYFP was detected in intercalated ducts, which do express *K19* but may have a lower level of expression than the cuboidal epithelial ducts. To our surprise, rare, yet detectable, acinar cells (<1%) and some islets cells (<1%) also express EYFP after TM administration (Fig. 2b,d). Co-IF staining with insulin antibodies or a ductal marker recognized by Dolichos biflorus agglutinin (DBA) confirmed that a small portion of the EYFP⁺ (positive) cells are endocrine cells (Fig. 2e). Although *K19* protein production in acinar or islet cells has not been shown, this low amount of recombination may be due to low, immunologically undetectable level of expression or to the CreER^T insertion cassette altering expression of the allele. Despite the leakiness in acinar and islet cells, our *K19^{CreERT}* mouse is the first inducible Cre line that allows for DNA recombination preferably in pancreatic ductal cells and it should complement other existing pancreas-specific Cre lines for pancreatic lineage and development-related studies. *K19^{CreERT}* does not induce recombination in all ductal cells as discovered by sampling multiple pancreatic sections in each of six adult pancreata. Yet this low activity is sufficient for many lineage and gene deletion studies. The lower *K19^{CreERT}* activity could create marked mutant cell clones in largely normal tissue/organs,

allowing cell-specific analyses without confounding effects from lethality or complete loss of tissue.

We determined whether *K19^{CreERT}* could induce recombination in the liver, stomach, the intestine, and the kidney (Fig. 3 and data not shown) by administering TM at 8 weeks of age and characterizing EYFP production after 1 week. TM induced widespread recombination in the epithelial portions of these organs (see Fig. 3). Only rare recombination events (~0.15% epithelial cells in six animals inspected) could be detected in the absence of TM in the stomach (Fig. 3d) and proximal intestinal epithelial cells (Fig. 3j) of the *K19^{CreERT}⁺; R26R^{EYFP}⁺* animals. Quantification showed that $30.8\% \pm 9.8\%$, $48.0\% \pm 19.5\%$, $54.1\% \pm 14.1\%$, $42.8\% \pm 14.4\%$, and $20.6\% \pm 6.8\%$ ($n = 6$) of the epithelial cells in the bile duct, duodenum, small intestine, large intestine, and stomach cells produced EYFP after TM administration, respectively. Within the kidney, recombination was also observed principally in the papillary ducts, with a lower amount of recombination in collecting ducts (data not shown).

We further examined whether *K19^{CreERT}* could label putative gut stem cells. Intestinal stem cells are thought to divide slowly giving rise to transient amplifying cells that rapidly divide to produce mature cells. However, label retention experiments indicate that most transient amplifying cells in the small intestine turn over approximately every 7 days (Potten, 1998). Therefore, by examining EYFP labeling 6 weeks after TM-induced cell marking, most labeled intestinal cells likely have arisen from intestinal stem cells. The identity and duration of transient amplifying cells in the stomach epithelium are not as well characterized. Yet, direct lineage tracing has suggested that each gastric gland is derived from one to three stem cells (Qiao *et al.*, 2007). Therefore *K19^{CreERT}*-based clonal analyses could likely reveal the presence of clone-forming cells in stomach as well.

K19^{CreERT}⁺; R26R^{EYFP}⁺ mice were administered with TM at P3 (0.1 mg TM) or at 8 weeks of age (4 mg TM). One or 6 weeks after TM injection, the EYFP-labeled cells in the whole gut were characterized. In non-TM treated animals ($n = 3$ at each stage), only a small number of single cell or small cell clusters (less than 20 cells) were observed to express EYFP (Fig. 4a,e). One week after TM injection, about 5% of all gut cells express EYFP in all animals, with some appeared as large EYFP⁺ cell clusters (data not shown). Six weeks after TM administration, large EYFP⁺ clones in all animals were frequently observed in TM-administered animals at P3 and 8 weeks of age (Fig. 4b–d,f–h, and data not shown). Several features of the EYFP⁺ clones suggest that they were derived from single gut stem cells. First, at the TM dosage utilized in this experiment, *K19^{CreERT}* only marked about 5% of all gut cells shortly (1 week) after TM administration. These marked cells appeared to distribute randomly in the intestinal epithelium, suggesting that it is unlikely to introduce DNA recombination simultaneously in multiple progenitor cells within a single crypt. Therefore, if all cells within a villus produced EYFP 6 weeks after TM administration, it is likely that this villus was derived from a marked stem cell. Second, EYFP⁺ clones containing multiple adjacent villus structures were frequently observed 6 weeks after TM administration, suggesting that each of these clones could arise from a single stem cell by expansion and fission during growth (Yen and Wright, 2006). Consistent with this notion, more than half of the large EYFP⁺ clones contained multiple villi or glands in animals treated with TM at P3. Most of the EYFP⁺ clones in animals treated with TM at adult ages only contain single villus or glands, indicating that the putative gut stem cells in the adults are less likely to expand than in neonatal stages. Third, all cells in some EYFP⁺ villi produced EYFP (Fig. 4d), suggesting that each villus was derived from a single, self-renewing EYFP⁺ cell, instead of a portion of several transient amplifying progenitor cells within a single villus. Similar EYFP-labeling features were observed in the stomach (Fig.

4g) after TM exposure, indicating the marking of putative gastric stem cells. These data suggest, but do not prove, that *K19^{CreERT}* could be used to label gut stem cells.

The non-essential nature of *K19* allowed us to derive *K19^{CreERT/CreERT}; R26R^{EYFP+}* animals. We found that 12 mg of TM administration to these mice at 8 weeks increased cell labeling to about 50–70% in the intestine (data not shown), about a 1.5-fold increase over that of *K19^{CreERT/+}; R26R^{EYFP+}* animals. Similar labeling enhancement in other organs was also observed. Notably, the percentage of labeled cells without TM administration increased as well to 0.4–0.7% ($n = 4$) in the duodenum. These data suggest that two doses of *K19^{CreERT}* could induce a higher recombination rate, more suitable for loss of gene function analysis.

In summary, we have demonstrated that the *K19^{CreERT}* mouse line can be utilized to introduce DNA manipulation in a variety of adult epithelial cells. Thus, it allows for temporally controlled gene recombination and cell lineage tracing during tissue regeneration in multiple cell types of endodermally derived organs.

MATERIALS AND METHODS

Mouse care and experimental procedures followed standard protocols in accordance with Vanderbilt animal usage guidelines. For initial knockout mice production, the C57BL/6 strain was used (Charles River Laboratories, Wilmington, MA). Subsequent strain maintenance and crosses utilized CD1 mice (Charles River Laboratories). *Flpe*, *R26R^{EYFP}* mice and genotyping methods were previously reported (Dymecki, 1996; Srinivas *et al.*, 2001). The *K19* targeting vector construction followed standard techniques. The final targeting vector contains the following DNA elements sequentially: 1.3 kb 5' *K19* arm-CreER^T-SV 40 PolyA-FRT-pGKneo-FRT-7.2 kb 3' *K19* arm. The *K19* recombination arms are derived from a BAC clone [RP23-217I3, purchased from the Children's Hospital of Oakland, Oakland, CA (Warming *et al.*, 2005)] using direct PCR or DNA recombineering, respectively. The oligos for PCR-amplifying the 5' arm: forward: 5'-GTTCGACCTTTGAGCTAGGAAGTGGT-3'; reverse: 5'-GAATTCGATGAGGAGGGAGACCAGAGCG-3'. The oligos for retrieving 3' arm: 5'-GGCCGCATAACTTCGTATAGCATACATTATACGAAGTTATTTAGGGCATAAAAA GCCACAGGTGAGGGCCTTGCTACTCCTCCTGCGGCCAGCAGTTCTCAG-3' and 5'-CCATGGCAGAAAGACCCCTCTCTACTCTCAACCCCATCTCTGTCCCCCGATCACC CCATAAACAACTCTCAGGCCTTTCCACCCTGCACACACAAAGAAAGGCTTCGTC TCACACTTC-3'. ES cell electroporation (129 SvEv-derived TL1 cell) and clone selection followed standard protocol. For ES cell screening, both PCR and Genomic Southern blot-based methods were used. First, a DNA oligo outside of the 5' arm (Pr1: 5'-AGGACTGACTTTGAACACTCTTC-3') and an oligo complementary to the 5'-end of CreER^T (Pr2: 5'-ATTTTGGTGTACGGTCAGTAAATT-3') were utilized to screen for the clones that had correct homologous recombination in the 5'-end of the *K19* locus. Targeted clones were expected to produce a 1.5-kb band, which is absent from nontargeted clones. For 3' recombination verification, a 1.4 kb DNA fragment outside the 3'-arm was PCR-amplified (forward: 5'-TCTTTCCATCTTCTTTTGGGA-3' and reverse: 5'-GCTCTGCCTCAACACTATGAG-3') and used for southern blot. If digested with Bam HI and Not I, the wild type or targeted allele produces a 14- or 12-kb band, respectively. Subsequent genotyping used PCR-based techniques. The oligos used are as follows: Pr3 (5'-GCAGAATCGCCAGGAATTGACC-3') and Pr2 (5'-GTTCTTGCGAACCTCATCACTC-3'). The unique band from the *K19^{CreERT}* gene is 300 bp. Mouse lines with the targeted *K19* allele were derived by standard blastocyst injection, using C57BL/6 mouse lines as acceptor. After germ line transmission was achieved, the FRT-flanked pGKneo selection cassette was deleted using a *Flpe* transgenic mouse line to

obtain the *K19^{CreERT}* allele (Dymecki, 1996). TM administration followed previous method (Gu *et al.*, 2002) with minor modification. Briefly, TM was dissolved in corn oil at 40 mg/ml. Desired dose was then injected subcutaneously into different-aged animals.

Immunohistochemistry followed standard protocols. Briefly, mouse tissues were fixed in 4% paraformaldehyde overnight at 4°C or 4 h at room temperature. Tissues were prepared as 6- μ m paraffin sections or 20- μ m frozen sections. Direct fluorescence observation was utilized to detect EYFP in frozen sections. Indirect staining was visualized within paraffin-embedded tissues using Rabbit anti-GFP antibody (ABCam, Cambridge, MA). Antibody binding was visualized with the Vectastain Elite ABC kit (Vector Labs, Burlingame, CA) followed by incubation in diaminobenzidine tetrahydrochloride substrate (Invitrogen, Carlsbad, CA). Colabeling was done with guinea pig anti-insulin and Cy5-conjugated donkey anti-guinea-pig IgG (Jackson ImmunoResearch, West Grove, PA) and with DBA-biotin (Sigma-Aldrich, St. Louis, MO) and Cy3-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). All antibodies utilized a 1:500–1:2,000 dilution.

For quantification of labeling efficiency, frozen tissue sections were utilized. Specifically, fixed tissues were prepared as 20- μ m frozen sections. One section per 200 μ m was collected. For counting YFP⁺ cells in the pancreatic ducts, DBA IF staining (as red fluorescence) was utilized to locate the duct. Pictures were taken at both green and red channels on same fields to compare EYFP⁺ and BDA-binding cells. The labeling efficiency was calculated as the area of EYFP⁺ cells/area of all epithelial tissues. For cell counting in other tissues, similar approaches were used, except that no immunofluorescence is required for visualizing the epithelial structures.

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FIG. 1.

K19 locus targeting strategy. (a) Structures of *K19* alleles and targeting vector. The targeting vector has a 1.3-kb 5'-arm, which locates immediately upstream of the *K19* ATG. The 3'-arm is a 7.2-kb fragment that has a 156-base pair overlap with the 5'-arm. The targeting vector uses Diphtheria Toxin A (DTA) subunit for negative selection and a FRT-flanked neomycin resistant gene for positive selection. Pr1 and Pr2 indicate the oligos utilized for genotyping. (b) Southern blots to show several targeted ES cell clones that give expected hybridization patterns (see Materials and Methods section for the expected size of DNA fragments). Sp, SpeI, Ba, BamHI, Sm, SmaI, Nc, NcoI, No, NotI.

FIG. 2. *K19^{CreERT}* allows inducible recombination in pancreatic duct cells. Panels (a–d) utilized antibodies to GFP/YFP that were then visualized colorimetrically (brown). Panels in (e) directly visualized yellow fluorescence emitted by the EYFP protein. (a) Control *K19-CreERT⁺; R26R^{EYFP}* pancreas without TM administration shows lack of any EYFP expression in the pancreas. Acinar, duct (green broken lines) and islets (red broken lines) were marked. (b–d) Pancreata of animals that received 12 mg TM at 8 weeks of age, 1 week before sacrifice. Note the abundance of labeled cells (red arrows) in ducts and a small number of labeled cells in islets and in acinar cells. Also note the presence of EYFP⁺ duct cells shown at high magnification (green arrows) in (c and d). (e) Pancreata of animals that received TM 1 day after birth and were sacrificed 1 week later. Even at this age, most labeled cells are ductal but there are rare labeled cells within islets (red arrows in e) and acini. Labeled cells were not randomly assorted in ducts; while the ducts shown have many labeled cells, other ducts have very few. In (e), immunofluorescence staining showed the presence of EYFP fluorescence (green) in DBA-reactive duct cells (red) and insulin⁺ β cells (blue) on a same microscopic field. Three separate channels (e1–e3) and a merge (e4) were shown. Bars = 50 μ m.

FIG. 3. *K19^{CreERT}* induces recombination in liver duct and gastrointestinal epithelial cells in the adults. Twelve milligram TM was administered at 8 weeks of age. EYFP expressions were analyzed after 1 week. EYFP production, detected by antibody staining (brown), was shown in the absence (a, d, g, and j) or presence (b, c, e, f, h, i, k, and l) of TM. (a–c) The liver. Note the both bile ductule cells (b) and intrahepatic bile duct cells (c) were labeled. (d–e) Stomach. (g–i) Large intestine. (j–l) Small intestine. Note that in the absence of TM, only stomach and small intestinal cells showed rare cells that express EYFP (black arrows). The red arrows in the liver sections point to the duct. Bars = 100 μ m.

FIG. 4. *K19^{CreERT}* labels putative gut stem cells. Direct fluorescence showed gut EYFP expression 6 weeks after 0.5 mg TM injection at P3. **(a–d)** Intestine. **(e–h)** Stomach. **(a and e)** No TM administration. Low magnification pictures were provided for a global view on the leakiness of *K19^{CreERT}* deleter. White arrows point to single cells. Red arrows point to two cell clusters. Insets in **(a)** and **(e)** are high amplification of single YFP⁺ cells costained with DAPI. EYFP, DAPI, and a merge channel were shown. **(b–d and f–h)** EYFP expression after TM administration. **(c)** and **(g)** are amplified areas (boxed) in **(b)** and **(f)**, respectively. **(d)** and **(h)** showed an intestinal crypt and a gastric gland, respectively. Cells were stained with DAPI to show nucleus of each cell. Note that in the crypt or gland (white broken lines), all cells are EYFP⁺. Bars = 40 μm.