Published in final edited form as:
Nat Methods. 2008 March ; 5(3): 227–229.

Tractable Cre-*lox* system for stochastic alteration of genes in mice

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Abstract

We developed a cell division–activated Cre-*lox* system for stochastic recombination of *loxP*-flanked loci in mice. Cre activation by frameshift reversion is modulated by DNA mismatch-repair status and occurs in individual cells surrounded by normal tissue, mimicking spontaneous cancer-causing mutations. This system should be particularly useful for delineating pathways of neoplasia, and determining the developmental and aging consequences of specific gene alterations.

Valuable mouse cancer models exist that combine conditional expression of the Cre recombinase with various *loxP*-flanked tumor suppressor or oncogene alleles[1]. In these model systems, 'cancer' is induced by gene alteration ubiquitously throughout the target tissue or in a selected cell type. But this does not accurately mimic the natural process of sporadic cancer initiation and progression. Two Cre-*lox* models that facilitate stochastic cancer gene alterations in isolated cells rely on homologous recombination to induce activation of an oncogene[2], or to induce inactivation of one or more tumor suppressor genes on the same chromosome[3]. The former system is restricted in that it is only applicable to activation of an oncogenic *K-ras* allele[2]. The latter more flexible system uses engineered *loxP-FRT* sites to induce mitotic recombination of individual chromosomes containing modified genes[3]. Here we report a highly versatile system that features Cre-mediated stochastic genetic changes in single cells or cell lineages in normal tissue. The system can be applied to any *loxP*-flanked allele, is dependent on cell division and can be modulated by DNA mismatch-repair status.

To construct an inactive but revertible *Cre* allele, we first engineered an 11-bp A·T run in a modified version of Cre[4] without altering the nuclear localization signal (Supplementary Methods online). We added an extra A·T base pair, creating a +1 bp out-of-frame *Cre* allele, which we termed *12A-Cre*. To ensure efficient expression at the intended target locus, we added a splice acceptor and internal ribosome entry site to *12A-Cre*. We cloned the modified *12A-Cre* plus a *neo* module between homology arms of the DNA mismatch repair gene *Pms2* (ref. 5) to generate a *Pms2-Cre* targeting vector. Targeting resulted in an out-of-frame *Cre* gene under the control of the *Pms2* promoter, which is expressed in several cell types, including stem cells of the mouse intestine[6]. Targeting to *Pms2* removed exon 2, creating a null allele, which we refer to as $Pms2^{cre}$ (Fig. 1a). Because of mismatch-repair deficiency, $Pms2^{cre/cre}$ mice should have increased frequency of −1 bp frameshifts[7] and hence increased *Cre* reversion relative to $Pms2^{cre/+}$ mice. Therefore, Cre activation frequency can be modulated appropriately for a particular study by breeding $Pms2^{cre/+}$ or $Pms2^{cre/cre}$ mice. Notably, this system should

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Published online at http://www.nature.com/naturemethods

better mimic sporadic carcinogenesis because Cre activation is stochastic, limited to individual cells and linked to cell division.

Because of our interest in intestinal cancers, we focused on Cre activation in the gastrointestinal tract. To estimate relative *Cre* reversion in *Pms2*$^{cre/+}$ and *Pms2*$^{cre/cre}$ mice, we bred them to *Cre*-inducible *Gt(rosa)26*^{tm1Sor} *lacZ* reporter mice (hereafter referred to as *Rosa26r*)[8]. We examined progeny over a 12-month period and visualized Cre activation in the intestine by β-galactosidase staining as 'ribbons' of blue staining up villus sides, or 'spots'[9]. As expected, *Cre* reversion in *Pms2*$^{cre/cre}$ (mismatch repair–deficient) mice was elevated ~100-fold relative to *Pms2*$^{cre/+}$ (mismatch repair–proficient) mice (Fig. 1). The average total numbers of spots in the *Pms2*$^{cre/+}$ and *Pms2*$^{cre/cre}$ mice were 26 and 3,300, respectively. We frequently observed a ribbon-like pattern of β-galactosidase staining from crypt base to the villus tip, with a greater number of blue spots in the proximal small intestine and trending toward fewer spots in the distal small intestine (Fig. 1). The total number of blue spots appeared to increase with age (Supplementary Fig. 1a online), consistent with mutations accumulating with age in the epithelium. Patch size was typically small, consistent with the progeny of a single stem or progenitor cell contributing to 1–3 villi (Fig. 1). This small patch size suggests stem cells with reactivated Cre usually remain confined to a single crypt with a single crypt supplying cells to several adjacent villi[10]. Typically, each of the four differentiated cell types in a particular crypt and associated villus appeared to stain positive for β-galactosidase (Fig. 1), consistent with *Cre* reversion having occurred in a stem cell. Microdissection of individual villi followed by a PCR assay for Cre-mediated recombination at the *Rosa26r* locus was consistently positive in blue-stained villi but negative in unstained villi (Supplementary Fig. 1b). Additionally, we observed patches of β-galactosidase–expressing (Cre-activated) cells in all tissues examined: intestine, pancreas, kidney, liver and muscle (data not shown), as expected based on the distribution of *Pms2* expression[6].

Numbers and sizes of β-galactosidase–positive patches provide a baseline indicator of intestinal stem cell fates after stochastic Cre reactivation. Each crypt contains multiple stem cells within a niche[10], and the inactivation or activation of a tumor suppressor or oncogene, respectively, may change the number of blue-staining cells by providing a selective advantage (more or larger patches) or disadvantage (fewer or smaller patches) to that stem cell, even in the absence of visible histological changes. In turn, clonal evolution, that is, the replacement of cell populations by the progeny of a single altered cell, within a normal appearing tissue can be visualized. As a next step, we tested the oncogenic allele, *LSL-K-ras*^{G12D} (ref. 11) activated by Cre-mediated excision of a stop codon, in our system. *Pms2*$^{cre/cre}$*, Rosa26r, LSL-K-ras*^{G12D} mice became moribund at 5 weeks of age because of high lung tumor burden, a phenotype previously associated with activation of *K-ras*^{G12D} (ref. 11). Lung tumors stained blue, consistent with Cre activation and subsequent *K-ras*^{G12D} expression (data not shown). We scored intestines from *Pms2*$^{cre/cre}$*, Rosa26r, LSL-K-ras*^{G12D} mice for the number and size of blue-staining regions (Fig. 2a). The numbers of blue 'spots' in the *Pms2*$^{cre/cre}$*, Rosa26r, LSL-K-ras*^{G12D} mice did not change, but the number of stained villi per patch, or blue patch size, increased in the small intestine and cecum (Fig. 2a,b). The morphology of most blue-staining villi was normal, with a few irregularly sized villi (Fig. 2c). The increase in patch size implies that sporadic activation of *K-ras*^{G12D} can confer a selective advantage at some stage, apparently by facilitating dominance of that stem cell over wild-type stem cells[12], including the ability to spread over larger areas, presumably through crypt fission[13]. This apparent clonal expansion and crypt fission of *K-ras*^{G12D}–expressing cells would facilitate the subsequent acquisition of the additional alterations needed to confer a visible neoplastic phenotype.

We developed a Cre-*lox* system that facilitates monitoring the short- and longer-term consequences of genetic changes in single cells in normal tissue in the mouse. This system relies on a stochastic reversion event, which activates Cre recombinase expression and can be

modulated by mismatch-repair status. Visualization of cell lineages experiencing Cre activation is facilitated by staining for Cre-inducible β-galactosidase, which also allows monitoring the relative survival and developmental capacity of mutant cells even in the absence of visible neoplasia. In this way, clonal evolution in normal-appearing tissues may be visualized. Patterns of β-galactosidase staining revealed that Cre activation can occur in stem cells and/or proliferating progenitors. Normally the progeny of a single stem cell are confined to a single crypt, but sporadic mutations may alter a stem cell's survival or ability to spread. Some mutations may be initially neutral, whereas others may be lethal or reduce fitness relative to wild-type stem cells within a niche. Alternately some mutations, as illustrated with *LSL-K-ras*^{G12D}, may confer a selective advantage, leading to expanded β-galactosidase–positive regions.

Because *Pms2*$^{cre/cre}$ mice are defective in mismatch repair, random mutations in the genome could complicate interpretations. However, controls can be studied in parallel, for example, mice that do not harbor tumor suppressor or oncogene Cre targets. Furthermore, although *Pms2*$^{cre/cre}$ mice have a reduced lifespan with a half-life of 10 months (data not shown), they can be useful for determining 'short–term' consequences of stochastic knockout of specific tumor suppressor loci individually, or of several loci in combination (unpublished data). Additionally, this system has inherent flexibility because either mismatch repair–proficient *Pms2*$^{cre/+}$ mice, which are not prone to cancer, or mismatch repair–deficient *Pms2*$^{cre/cre}$ mice can be used to modulate the activation rate of *Cre*. Judicious use of *Pms2*$^{cre/+}$ or *Pms2*$^{cre/cre}$ mice will be directed by the nature and number of Cre targets (*loxP*-flanked alleles) in a particular study.

Because *Pms2* is expressed in multiple tissues and mismatch repair is likely to be active in a variety of stem cell types, the *Pms2*^{cre} system described here offers flexibility in terms of probing both normal and abnormal development in a variety of tissues. In addition, having a widely expressed promoter driving *Cre* offers the potential to determine for any given tumor whether *Cre* activation occurring in the tissue or cell type that gave rise to the tumor and/or an underlying or surrounding tissue type was critical. Nevertheless, it may be advantageous in certain situations to use a tissue-specific promoter, such as the villin promoter, which would target Cre activation to the epithelium of the mouse gut[14].

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

We thank M. Wong, N. Erdeniz, J. Johnson, O. Reilly and K. MacDonald for critically reading the manuscript. This work was supported by US National Institutes of Health grants R37 GM32741 to R.M.L. and 2 RO1 CA 80077 to D.S.

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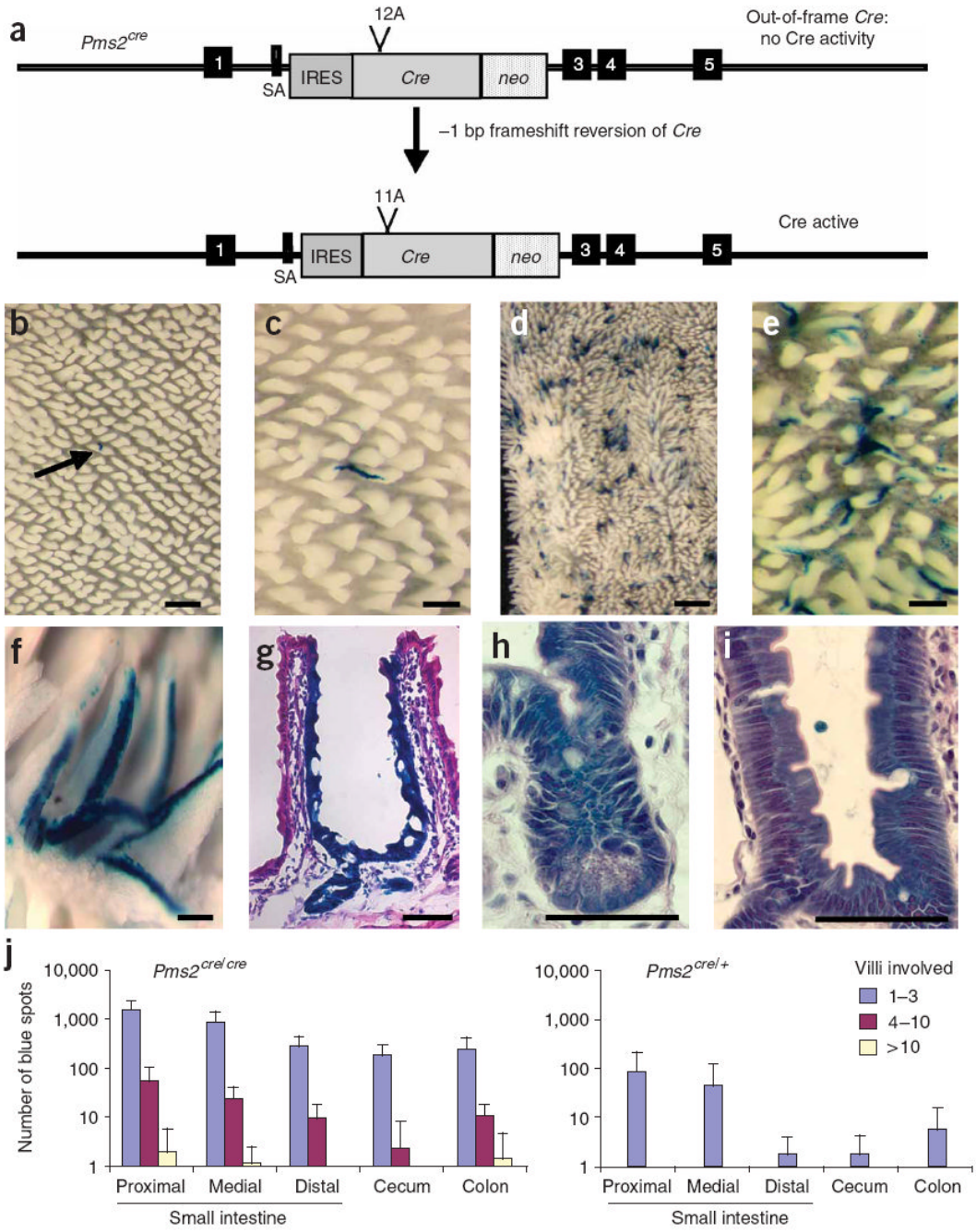

Figure 1. Generation and characterization of *Pms2*^{cre} mice

(**a**) A schematic of the *Pms2*^{cre} allele activation by a −1-bp frameshift in the 12A run. SA, splice acceptor; IRES, internal ribosome entry site. Boxes 1 and 3–5 represent exons.

(**b–f**) β-galactosidase staining in whole-mount mouse intestinal sections. Different magnification images of *Pms2*$^{cre/+}$ (**b,c**) and *Pms2*$^{cre/cre}$ (**d–f**) small intestine. Scale bars, 1 mm (**d**), 0.5 mm (**b**), 0.25 mm (**c,e**) and 0.1 mm (**f**). Arrow in **b** indicates the single spot in the field.

(**g–i**) Hematoxylin and eosin–stained 5-μm paraffin sections of *Pms2*$^{cre/cre}$ small intestine crypt. Scale bars, 50 μm (**g**) and 25 μm (**h,i**).

(**j**) Distribution of average number of blue spots in the gastrointestinal tract of age-matched *Pms2*$^{cre/cre}$ (n = 10) and *Pms2*$^{cre/+}$ (n = 4) mice. Error bars, s.d.

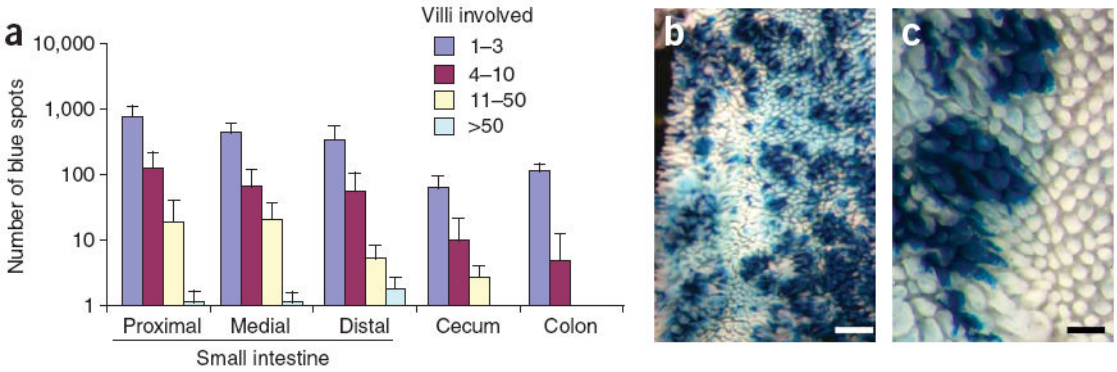

Figure 2. Activation of *K-ras* results in larger regions of β-galactosidase staining

(**a**) Distribution of average spots per region in *Pms2*$^{cre/cre}$, *LSL-K-ras*^{G12D} mice (n = 5). Error bars, s.d. The size spot per area increases compared to *Pms2*$^{cre/cre}$, *Rosa26r* mice. (**b,c**) β-galactosidase staining in whole-mount small intestine from *Pms2*$^{cre/cre}$, *LSL-K-ras*^{G12D} mice shows expanded (large) regions of staining. Scale bars, 1 mm (**b**) and 0.5 mm (**c**).