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Generating somatic mosaicism with a Cre recombinase– microsatellite sequence transgene

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Abstract

Strategies for altering constitutional or somatic genotype in mice are well established, but approaches to generate mosaic genotypes in mouse tissues are limited. We showed that a functionally inactive Cre recombinase transgene with a long mononucleotide tract altering the reading frame was stochastically activated in the mouse intestinal tract. We demonstrated the utility of this approach by inducing colonic polyposis after Cre-mediated bi-allelic inactivation of the *Apc* gene.

Mouse transgenic and knockout models offer definitive insights into vital biological processes and model features of human diseases. Transgenic mouse strains expressing unique cellular oncogenes or DNA tumor virus oncogenes in specific tissues have been generated to model cancer development. Homologous recombination approaches in embryonic stem cells have allowed analysis of mice carrying constitutional loss-of-function mutations in key tumor suppressor genes. Use of defined regulatory elements to direct expression of Cre recombinase transgenes to specific tissues permits conditional activation of cellular oncogenes or inactivation of tumor suppressor genes and has allowed pursuit of an even greater range of studies of the cancer process^{1,2}. The introduction of Cre-expressing adenoviruses to alter genotype in a subset of somatic cells has been described^{3–5}, but virus introduction into internal organs can be technically challenging. Hence, most studies have emphasized work in which oncogene activation and/or tumor suppressor gene inactivation is generated in essentially all somatic cells of one or more tissues. Certain recombinase-dependent mitotic recombination approaches are useful for generating rare homozygous mutant cells in a background of heterozygous mutant cells^{6,7}. Nonetheless, nearly all mouse genetic models described to date most closely reflect the inherited cancer setting in man, whereas most human cancers are thought to arise from cycles of somatic mutation and clonal selection, promoting outgrowth of rare variant clones in a sea of wild-type cells.

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AUTHOR CONTRIBUTIONS

A.A., T.H., Y.F. and G.T.B. performed experiments. T.M.G. provided key reagents. E.R.F. supervised the work and wrote the manuscript.

Given this background, we sought to develop an approach to activate Cre recombinase expression in a subset of somatic cells in a particular tissue. Our prior studies revealed that mice carrying transgenes regulated by a 9.5-kb fragment containing human *CDX2* homeobox gene promoter and upstream flanking elements (*CDX2P9.5*) showed broad transgene expression throughout the caudal region of the embryo during early development, with tightly restricted transgene expression in the distal small intestine, cecum and colon during late gestation and in adult tissues⁸. It is well known that certain microsatellite sequence tracts, including mono-, di- or tri-nucleotide repeats, are mitotically unstable in mammalian somatic cells, particularly when cells lack wild-type mismatch repair activity^{9,10}. Hence, we assessed whether a *CDX2P9.5*-regulated Cre transgene with a long mononucleotide tract between its initiating methionine (ATG) codon and the remainder of the Cre-coding region might be susceptible to somatic mutation in mouse tissues. To address this possibility, we generated Cre recombinase transgenes with either 19 or 22 guanine nucleotides (*G19Cre* or *G22Cre*, respectively) introduced downstream of the initiating ATG codon (Fig. 1a and Supplementary Methods online). These guanine insertions disrupted the reading frame and provided ideal expression levels (Supplementary Fig. 1 online and data not shown). We anticipated that some frameshift mutations in the guanine nucleotide tract would create Cre alleles that were read in frame in a subset of somatic cells, with resultant expression of a functional Cre protein.

We generated 8 independent transgenic mouse lines carrying the *CDX2P9.5-G19Cre* or *CDX2P9.5-G22Cre* alleles (Supplementary Table 1 online) that transmitted the transgene alleles to progeny. We crossed these 8 lines to a well-characterized reporter mouse line (R26R) with a β -galactosidase (β -gal) reporter gene inserted in the *Gt(ROSA)26Sor* (also known as *ROSA26*) locus¹¹, and looked for somatic activation of Cre recombinase in a subset of caudal-derived cells known to express *CDX2P9.5*-regulated transgenes⁸. We found a small fraction of the cells in the tail of one line of transgenic embryos (line 189) that showed Cre-mediated β -galactosidase (β -gal) expression (Fig. 1b). This pattern of limited Cre recombinase activity in a few caudal-derived cells in the line 189 transgenic mice contrasts with broad Cre transgene expression and function during early development in the caudal half of mice carrying constitutively active *CDX2P9.5-NLSCre* transgenes⁸. Based on Southern blot analysis, line 189 mice carry about 80 copies of the transgene (data not shown). Analysis of other independent transgenic lines carrying the *CDX2P9.5-G22Cre* or *CDX2P9.5-G19Cre* transgene revealed some lines with broader activation of the Cre transgene during caudal region development (for example, line 91; Supplementary Fig. 2 online), but other lines showed either essentially constitutive Cre expression throughout all caudal-derived tissues or no detectable Cre expression (Supplementary Table 1). Our studies did not reveal a consistent relationship between transgene copy number and the pattern of Cre activation, perhaps because of the limited number of lines examined and in part because the site of transgene insertion might be important in influencing transgene expression. Because line 189 mice transmitted the *CDX2P9.5-G22Cre* transgene to roughly 50% of offspring and the mice reproducibly showed restricted activation of Cre function in a fraction of caudal-derived cells during development, we used line 189 mice for all further studies.

Because of our prior data showing *CDX2-NLSCre* transgene expression in the distal small intestine, cecum and colon of adult mice⁸, we carried out immunohistochemical analysis of β -gal expression in the intestinal tract of *CDX2P9.5-G22Cre;R26R* mice. We detected many independent foci of β -gal expression in the cecum and colon of a 6-week-old mouse, with the most frequent activation of β -gal expression occurring in cecum and proximal colon (Fig. 1c). Higher-resolution microscopic analysis indicated single crypts and multiple adjacent crypts showed β -gal expression, with apparent single-positive crypts in distal colon. Analysis of β -gal enzymatic activity in various tissues from a 4-week-old transgenic mouse yielded data for the intestinal tract confirming *in situ* analyses, with no evidence of extra-intestinal activation of β -gal expression, except for the tail (Fig. 1d and data not shown). The pattern of β -gal

activation observed in line 189 transgenic mice appears to be consistent over multiple generations, as we observed essentially identical patterns through generation F3 (data not shown).

Studies of β -gal expression in the gastrointestinal tract of *CDX2P9.5-G22Cre;R26R* mice of different ages indicated greater numbers of β -gal-expressing foci in the distal small intestine, cecum, and proximal and distal colon of older mice (Supplementary Fig. 3 online). The size of the β -gal-positive patches in both ileum and colon also appeared larger in the older mice (Supplementary Fig. 3). The larger patch size may reflect crypt fission events, wherein a β -gal-expressing intestinal or colon stem cell in a crypt undergoes a symmetric division, leading to production of two β -gal-positive stem cells and subsequent establishment of a new β -gal-positive crypt adjacent to the initial parent crypt. Additional crypt fission cycles would generate larger β -gal-positive patches. Attempts to directly demonstrate somatic frameshift mutations in the G22 tract in colon epithelial tissues from line 189 mice were unsuccessful, likely because the high copy number of the *CreG22* transgene in line 189 mice coupled with known polymerase ‘stutter’ in PCR of long mononucleotide sequence tracts made it technically unfeasible to detect the one or a few transgene alleles that might have been activated by mutation in a background of roughly 80 total alleles.

To address the utility of the *CreG22* transgene for modeling colon tumor development, we crossed transgenic *CDX2P9.5-G22Cre* mice to mice carrying adenomatous polyposis coli (*Apc*) alleles with *loxP* sites flanking exon 14 (*Apc^{flox/flox}*). Cre-targeting of the *Apc^{flox}* allele deletes exon 14, leading to a frameshift mutation at codon 580 and a truncated APC protein. Our prior studies had revealed embryonic lethality in all mice homozygous for the *Apc^{flox}* allele that carried a constitutively active Cre transgene under control of the *CDX2P9.5* elements⁸. We had also previously found that *CDX2P9.5-NLSCre;Apc^{flox/+}* mice were viable and developed 6–10 distal colon adenomas by the age of 300 d, albeit with a few small intestine, cecal and proximal colon adenomas as well⁸. In our studies described here, we found that *CDX2P9.5-G22Cre* transgenic mice homozygous for the *Apc^{flox}* allele were viable but lived only for 10–27 d after birth, whereas *CDX2P9.5-G22Cre* transgenic mice heterozygous for the *Apc^{flox}* allele had no overt signs of disease through 200 d of age (Fig. 2a and data not shown). The proximal colon of *CDX2P9.5-G22Cre;Apc^{flox/flox}* mice revealed large numbers of polypoid lesions (Fig. 2b), which, when examined microscopically, showed dysplastic (adenomatous) changes. The neoplastic epithelium showed prominent β -catenin staining in the cytoplasm and nucleus (Fig. 2c). Our data showing that bi-allelic *Apc* inactivation in mouse colon epithelium leads to very rapid development of adenomatous lesions are consistent with the pre-eminent role assigned to *APC* inactivation in human colon adenoma development¹². Given the limited time between *Apc* inactivation and adenoma development, our findings imply bi-allelic *Apc* inactivation is sufficient on its own to instigate adenomatous changes in mouse colon epithelium, without a requirement for additional genetic events. Death in all *CDX2P9.5-G22Cre;Apc^{flox/flox}* mice appeared to be due to florid polyposis in the proximal colon and cecum, though the mice often displayed minor tail malformations (Supplementary Fig. 4 online).

Our strategy for generation of mosaic activation of Cre transgene function preferentially in the colon should be applicable in other mouse tissues and for other genes. A similar microsatellite sequence strategy to the one described here was used to modify placental alkaline phosphatase, allowing studies of frameshift mutation rates *in vivo*^{13,14}. We predict mosaic activation of microsatellite-containing transgenes would likely be seen in tissues undergoing considerable proliferative expansion during embryonic development or in proliferative adult tissues, such as the skin, bone marrow and perhaps other organs (for example, uterus), or in adult tissues that undergo marked proliferation after injury (for example, liver regeneration). The frequency of somatic mutation in transgenes containing microsatellite tracts may depend on multiple

variables, such as the length and sequence content of the repeat tract, transgene copy number, transgene insertion site in the genome, genetic background of the strain, dietary and environmental exposures, and tissue-specific differences in DNA repair. In addition to the potential value in modeling pathological states such as cancer, mosaic transgene activation may be useful for studies of cell lineage and the role of specific genes in physiological processes, especially cell-fate determination, proliferation, and survival and tissue morphogenesis, where the phenotype of adjacent mutant and wild-type clones can be directly compared *in vivo*.

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Figure 1. *CDX2P9.5-G22Cre* transgenic mice and somatic activation of Cre function. **(a)** Schematic representation of *CDX2P9.5-G22Cre* transgene construct. **(b)** A *CDX2P9.5-G22Cre* transgenic embryo (17.5 d post-coitum) from a homozygous R26R reporter female mouse crossed with an F1 *CDX2P9.5-G22Cre* transgenic male (line 189). Specific β -gal staining was seen only on the tip of the tail (top right) as compared with the absence of β -gal staining in wild-type embryo (bottom right). Scale bars, 2 mm. **(c)** X-gal staining of gastrointestinal tract tissues from a 6-week-old *CDX2P9.5-G22Cre;R26R* mouse. Stomach, followed by small intestine, cecum and colon, with anus at lower right (left panel). Stereomicroscope images of β -gal staining, with arrows indicating the cecal, proximal colon and distal colon regions visualized (middle). Cryostat sections of tissue from cecum, proximal colon and distal colon stained with X-gal (right). Scale bars, 1 cm (left), 1 mm (middle) and 100 μ m (right). **(d)** Results of a quantitative β -gal assay of tissues comparing β -gal enzymatic activity in tissues from a 4-month-old *CDX2P9.5-G22Cre;R26R* mouse versus a control R26R mouse.

Figure 2.

Polyposis in cecum and proximal colon in *CDX2P9.5-G22Cre;Apc^{flox/flox}* mice. **(a)** Survival rates in *CDX2P9.5-G22Cre;Apc^{flox/flox}* mice (red; $n = 10$) compared to *CDX2P9.5-G22Cre;Apc^{flox/+}* mice (black; $n = 6$). **(b)** Dissected whole gastrointestinal tract of *CDX2P9.5-G22Cre;Apc^{flox/flox}* mouse with extensive polyposis in cecum and proximal colon (left), with a higher power view of proximal colon polyposis (right). Scale bars, 1 cm (left) and 5 mm (right). **(c)** Histological analysis of hematoxylin and eosin–stained section from proximal colon of a *CDX2P9.5-G22Cre;Apc^{flox/flox}* mouse, with tubular adenomatous glands replacing normal mucosa, and higher power hematoxylin and eosin–stained region of adenomatous glands (top right). Analysis of nuclear β -catenin staining in neoplastic cells (bottom right). Scale bars, 200 μ m (left) and 25 μ m (right).