Inducible activation of Cre recombinase in adult mice causes gastric epithelial atrophy, metaplasia, and regenerative changes in the absence of “floxed” alleles

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Huh WJ, Mysorekar IU, Mills JC. Inducible activation of Cre recombinase in adult mice causes gastric epithelial atrophy, metaplasia, and regenerative changes in the absence of “floxed” alleles. Am J Physiol Gastrointest Liver Physiol 299: G368–G380, 2010. First published April 22, 2010; doi:10.1152/ajpgi.00021.2010.—The epithelium of the mammalian gastric body comprises multiple cell types replenished by a single stem cell. The adult conformation of cell lineages occurs well after birth; hence, study of genes regulating stem cell activity is facilitated by inducible systems for gene deletion. However, there is a potential pitfall involving the commonly used inducible Cre recombinase system to delete genes: we report here that induction of Cre using standard doses of tamoxifen led to marked spasmolytic polypeptide-expressing metaplasia of the stomach within days and profound atrophy of the entire epithelium with foci of hyperplasia by 2 wk even in the absence of loxP-flanked alleles. Cre induction caused genotoxicity with TdT-mediated dUTP nick-end labeling (TUNEL)-positive apoptosis (TUNEL-positive cells) and increased levels of DNA damage markers (γH2AX, p53, DDIT3, GADD45A). Although Cre was expressed globally by use of a chicken actin promoter, the effects were almost entirely stomach specific. Despite severe injury, a subset of mice showed near complete healing of the gastric mucosa 11–12 wk after Cre induction, suggesting substantial gastric regenerative capacity. Finally, we show that nongenotoxic doses of tamoxifen could be used to specifically delete loxP-flanked Bmpr1a, the receptor for bone morphogenetic protein 2, 4, and 7, causing antral polyps and marked antral-pyloric hyperplasia, consistent with previous reports on Bmpr1a. Together, the results show dose-dependent, potentially reversible sensitivity of the gastric mucosa to Cre genotoxicity. Thus we propose that tamoxifen induction of Cre could be used to induce genotoxic injury to study the regenerative capacity of the gastric epithelial stem cell.

CRE A SITE-SPECIFIC RECOMBINASE FROM BACTERIOPHAGE P1 RECOMBINES DNA BETWEEN TWO 34-BASE PAIR loxP SITES, EACH OF WHICH CONSISTS OF TWO 13-BASE PAIR INVERTED REPEATS SEPARATED BY AN 8-BASE PAIR CORE REGION (37). THE CRE-loxP SYSTEM IS WIDELY USED TO ACTIVATE OR INACTIVATE GENE EXPRESSION IN A TISSUE- AND/OR TIME-SPECIFIC MANNER.

However, it has been reported that mammalian genomes contain frequent cryptic loxP sites (38) with an overall frequency estimated to be 1.2 per megabase (34). Accordingly, it has been shown that Cre recombinase can induce apoptosis, growth inhibition, and chromosomal aberration in mammalian tissue culture cells (18) even in the absence of alleles flanked by exogenous, “true” loxP sites. Genotoxicity induced by Cre recombinase has been shown to be dose dependent, and an optimal effective dosage of Cre recombinase that permits true loxP site-specific recombination without cryptic-loxP-associated toxicity was titratable (1, 18).

Given the widespread use of Cre-loxP systems for analyzing loss or gain of function of genes in mice, the caveats associated with using Cre are important. Recently, several studies have shown that ubiquitous or tissue-specific Cre recombinase (24) expression can cause toxicity during development in vivo even in mice without gene-specific floxed alleles (7, 10, 15, 32). However, the toxic effects of inducing Cre expression in an adult animal have not been detailed.

The epithelium of the mammalian gastric body (corpus) can undergo aberrant differentiation (metaplasia) in response to certain types of injury (11, 20). For example, in certain patients infected with Helicobacter pylori, gastric units undergo transformation wherein acid-secreting parietal cells are lost, and digestive enzyme secreting chief (zymogenic) cells begin to reexpress genes whose expression is normally restricted to mucous neck cells, the cell progenitor (6, 29, 31). This type of metaplasia is known as spasmolytic polypeptide-expressing metaplasia (SPEM), because the mature zymogenic cells begin to reexpress the neck cell gene spasmolytic polypeptide (aka Trefoil factor 2, TFF2) while maintaining expression of zymogenic genes (e.g., pepsinogens and, in mice, gastric intrinsic factor) (8, 28). In mice, SPEM can be induced by Helicobacter species (27) or by direct ablation of parietal cells by either a transgenic (6, 16) or drug approach (29). SPEM-type metaplasia predisposes patients for subsequent development of gastric adenocarcinoma (33). Induction of gastric metaplasia by a genotoxic approach has not been reported.

Here we show that inducible Cre recombinase controlled under a universally expressed promoter caused reproducible genotoxicity in adult mouse stomach. Induction of Cre caused increased apoptosis and markers of DNA damage within 3 days, diffuse SPEM within a week, and diffuse atrophy with focal hyperplastic regenerative changes in 2 wk. Among all organs examined, the stomach is uniquely affected by Cre genotoxicity; however, the gastric mucosa shows considerable regenerative potential since mice at 11–12 wk following Cre induction survived and showed substantial recovery. Toxic effects were tamoxifen dose dependent. Thus we were able to titrate an optimal dosing schedule that induced loxP site-specific recombination without nonspecific Cre toxicity and used this dose to induce global deletion of floxed Bmpr1a (also known as Alk3, the receptor for bone morphogenetic protein (BMP) 2, 4, and 7). Mice lacking BMPR1A showed general-
ized alopecia and epithelial hyperplasia with hyperplastic polyp formation in the gastric antrum.

MATERIALS AND METHODS

Animals. All experiments involving animals were performed according to protocols approved by the Washington University School of Medicine Animal Studies Committee. Mice were maintained in a specified-pathogen-free barrier facility under a 12-h light cycle. To make tamoxifen-inducible \textit{Bmpr1a} knockout mice, \textit{Bmpr1a\textsuperscript{lox/lox}} mice (22) were crossed with \textit{CAGGCreERT\textsuperscript{Tm}} transgenic mice (12), which express Cre \textit{ERT\textsuperscript{Tm}} under the control of a modified chicken \(\beta\)-actin promoter. To evaluate efficiency of recombination, \textit{CAGGCreERT\textsuperscript{Tm}} mice were crossed with a R26R reporter line (35), which expresses floxed \(\beta\)-galactosidase under the control of the Rosa26 promoter. R26Cre\textit{ERT\textsuperscript{Tm}} mice were described previously (40). Tamoxifen (ranging from 0.75 to 5 mg per 20 g body wt, Sigma, St. Louis, MO) was injected intraperitoneally for 3–7 consecutive days to activate Cre recombinase. Tamoxifen was dissolved in 10% ethanol and 90% sunflower seed oil (Sigma) at 1 mg/ml. Vehicle (100% ethanol and 90% sunflower seed oil) was injected intraperitoneally as a control.

\textbf{Genotyping.} Tissue was lysed with 25 mM sodium hydroxide (pH 12.0) at 95°C for 25 min. Then the solution was neutralized with the same volume of 40 mM Tris buffer (pH 5.0) as the volume of sodium hydroxide. This solution was used for PCR. For Cre and \textit{Bmpr1a}, PCR was done with RedTag (Sigma), and KlenTag (DNA Polymerase Facility, Washington University, St. Louis, MO) was used for LacZ PCR. Primers were as follows: Cre forward AGG GAT CGC CAG GCG TTT TC and reverse GGG AGA TAC ACT TGC TGA and gene-specific primers on an Mx3000P (Stratagene, La Jolla, CA). For \textit{Bmpr1a} alleles, we noticed profound remodeling of the corpus gastric epithelium (Fig. 1B). Parietal cells atrophy, and cellular proliferation, measured by BrdU incorporation, was markedly increased (Figs. 1B, 2A). Nonapoptotic gastric glandular cells showed coexpression of both neck progenitor cell (bound by the lectin GS-II) and zymogenic cell progenitor cell (bound by the lectin GS-II) and zymogenic cell

\textbf{RESULTS}

\textit{Induction of Cre recombinase causes metaplasia and destruction of most gastric epithelial cells in the absence of floxed alleles.} We initially set out to investigate the role of BMP signaling in the adult stomach and bred mice that had floxed \textit{Bmpr1a} alleles and carried a \textit{CAGGCreERT\textsuperscript{Tm}} transgene that expressed globally in all adult tissues. Injection of tamoxifen in these mice would be expected to delete the \textit{Bmpr1a} gene by specific recombination of the flanking \textit{loxP} sites. In control experiments, we bred \textit{CAGGCreERT\textsuperscript{Tm}} mice that did not have floxed \textit{Bmpr1a} alleles. To our surprise, as early as 3 days after tamoxifen injection, in either the \textit{CAGGCreERT\textsuperscript{Tm}} mice with floxed alleles or those without, we noticed profound remodeling of the corpus gastric epithelium (Fig. 1B). Parietal cells atrophy, and cellular proliferation, measured by BrdU incorporation, was markedly increased (Figs. 1B, 2A). Nonapoptotic gastric glandular cells showed coexpression of both neck progenitor cell (bound by the lectin GS-II) and zymogenic cell

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Fig. 1. Metaplasia, atrophy, and hyperplasia caused by Cre recombinase in mouse stomach mucosa. Mice expressing transgenic CAGGCreERTM and no floxed alleles at 0 (A), 3 (B), 7 (C), and 14 days (d) (D and E) following intraperitoneal injection of tamoxifen, which activates recombinase activity by shuttling Cre recombinase into the nucleus (E is a section taken nearer the pyloric sphincter, whereas D is more proximal). F: wild-type mouse 14 days following intraperitoneal injection of tamoxifen. Wild-type mouse stomach treated with tamoxifen shows normal mucosal epithelium, indicating that phenotypes shown in D and E are not caused by tamoxifen treatment. Insets to B: orange box shows a higher magnification of an apoptotic parietal cell (orange arrowhead; note eosinophilic cytoplasm and hyperchromatic nuclear fragment); green box shows a mitotic figure (white arrowhead). Left bottom: higher magnification of boxed area in E. Right bottom: higher magnification photomicrographs of regions at left bottom. Note abundant mitotic figures in this regenerative focus (white arrowheads). Scale bar = 50 μm for A–D, and F (units oriented with lumen to left), 500 μm for E. Note progressive atrophy of gastric epithelium, so that by day 14 following Cre recombinase activation there is diffuse mesenchymal edema with a single cell epithelial layer (black arrowhead) covering much of the mucosa with no remaining gastric glands, only scattered regenerative foci.
By day 7, the SPEM pattern of differentiation with absent parietal cells (Fig. 1C), increased proliferation, and coexpression of GIF and GS-II predominated throughout the body of the stomach (data not shown). In addition, numerous glandular cysts formed, and mesenchyme around gastric units expanded (Fig. 1C). At 14 days after tamoxifen injection, nearly the entire epithelium was replaced by stromal cells. The vast majority of the residual epithelium was highly atrophic, comprising a single cell layer overlying an expanded edematous stroma. There were occasional regenerative foci consisting of tortuous glandular structures composed of highly proliferative (BrdU-positive) cells (Figs. 1, D, and E, and 2C). These patterns were consistently observed in eight separate experiments, and in a total of 69 mice. Given the dramatic phenotypes even in the absence of floxed alleles, we confirmed the genotypes in many of these mice by two independent rounds of tail genotyping and then, finally, by DNA extracted directly from the paraffin-embedded tissue sections showing the lesions. The observed gastric phenotypes were produced only in stomachs bearing CAGGCreERTM alleles after tamoxifen injection; tamoxifen injection into wild-type mice did not show this pattern of epithelial cell loss and stromal cell replacement (Fig. 1F), precluding tamoxifen alone as the cause of the phenotype and confirming that Cre recombinase activity was required for the aberrant differentiation.

The gastric phenotype is caused by Cre genotoxicity. Cre recombinase can cause DNA damage which leads to DNA

![Figure 2](http://ajpgi.physiology.org/)

**Fig. 2.** Increased proliferation and spasmolytic polypeptide-expressing metaplasia (SPEM) induced by Cre recombinase. A: photomicrographs of isthmal (proliferative) zone with surface pit cells (purple with AAA lectin; left) and mucous neck cells (red with GS-II lectin; right) taken 3 days following vehicle injection (left) and tamoxifen injection (right) in CAGGCreERTM mice. Note increase in bromodeoxyuridine (BrdU; green; e.g., white arrowheads)-positive nuclei in tamoxifen-treated CAGGCreERTM mice, indicating increased proliferation. B: neck and base zones of gastric units from 3 days following vehicle injection (left) and tamoxifen injection (right) in CAGGCreERTM mice with neck (GS-II, green labeling neck cells) to left and base to right [anti-gastric intrinsic factor (GIF), red, labeling zymogenic cells]. Note that in gastric units with induced Cre recombinase, markers of neck and zymogenic cells are coexpressed in individual cells, indicative of SPEM, whereas in wild-type gastric units neck and zymogenic cells are largely distinct. C: bases of regenerative foci in CAGGCreERTM mice 14 days following Cre recombinase activation by tamoxifen. Proliferative cells (BrdU-positive, purple nuclei; e.g., arrowheads) are abundant and overlapping, and neck cell markers are expressed diffusely in the base (green, GS-II) with varying degrees of coexpression of the zymogenic cell marker (GIF, red). Scale bars indicate magnifications for all panels in A, B, and C.
repair, cell cycle inhibition, and/or cellular apoptosis. Consistent with genotoxicity as a mechanism for the gastric phenotype following induction of Cre recombinase, we observed an increase in apoptosis in CAGGCreERTM stomachs after tamoxifen injection compared with controls (Fig. 3A). Also, the DNA double-strand break marker, γH2AX, was positive only in CAGGCreERTM mouse stomachs after tamoxifen injection (Fig. 3B). This was confirmed with Western blot (Fig. 3C). Finally, we assessed levels of several DNA damage-responsive transcripts: p53, Ddit3, and Gadd45a expression levels were statistically significantly (P < 0.05 to <0.01) increased in CAGGCreERTM stomachs after tamoxifen injection compared with controls (Fig. 3D).

**Cre recombinase induces genotoxicity specifically in the stomach and not other organs.** Because CreERTM expression in our experiments was driven by a β-actin promoter with relatively universal expression, all cells could theoretically be affected by Cre recombinase following tamoxifen treatment. We tested other organs (small intestine, large intestine, liver, pancreas, spleen, urinary bladder, lung, and skin) for Cre induced changes. All other organs in tamoxifen-treated CAGGCreERTM mice were indistinguishable from controls [Fig. 4, A–D; n = 31 mice in 5 experiments; see also Mysorekar et al. (23) for specific analysis of bladder] with the exception of small intestine, which showed mild, focal crypt hyperplasia with an increase in crypt apoptotic figures (Fig. 4A). We assessed levels of DNA-damage-responsive tran-

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**Fig. 3. Genotoxicity induced by Cre recombinase.** A: TdT-mediated dUTP nick-end labeling (TUNEL; green) labeling of wild-type mice 7 days (left) as well as CAGGCreERTM mice 3 days (middle), and 7 days following tamoxifen injection. Neck and/or SPEM cells are labeled with GS-II (purple). Note abundant TUNEL-positive nuclei (e.g., arrowheads) indicating diffuse cell death in mice with Cre recombinase activated. B: CAGGCreERTM mice 3 days following injection of vehicle alone (left) and tamoxifen (right). Note increased genotoxicity in Cre recombinase activated, tamoxifen-treated mice with increased nuclei positive for γH2AX (purple; e.g., green arrowheads), indicating DNA damage. GS-II is green, GIF is red. C: Western blot showing increase in γH2AX in 3 days following tamoxifen treatment in 3 different CAGGCreERTM mice relative to 2 different control mice. Anti-α-tubulin of same lanes was used as loading control. D: abundance in whole stomach of DNA damage transcripts measured by quantitative RT-PCR (qRT-PCR) for each of the indicated genes is expressed as a ratio of 3 days following tamoxifen-treated CAGGCreERTM mice relative to control. Note in all figures: qRT-PCR expressed as Log2 scale. *P < 0.05; **P < 0.01.
scripts by qRT-PCR and also found no difference between tamoxifen-treated CAGGCreERTM mice and tamoxifen-treated wild-type controls with the exception of liver, which showed increase in p53, Ddit3, and Gadd45a expression (data not shown).

Cre genotoxicity positively correlates with the Cre transgene expression level. We next performed experiments to test whether stomach specificity of the genotoxicity induced by CAGGCreERTM was due to higher levels of Cre transgene expression in the stomach relative to other organs. qRT-PCR showed that, in CAGGCreERTM mice, stomach had the second highest constitutive levels of Cre transgene expression in various organs in CAGGCreERTM mice using qRT-PCR. Stomach has the highest Cre recombinase expression level except liver (n = 3 animals/group; P < 0.07 for stomach vs. small intestine). *P < 0.05; ***P < 0.001.

Fig. 4. Other organs are not affected by Cre as severely as stomach. Small intestine (A), large intestine (B), liver (C), and pancreas (D) from vehicle-treated (top) and tamoxifen-treated (bottom) CAGGCreERTM mice 7 days following injection. Note that organs with activated Cre recombinase do not differ substantially from control, although there is mild crypt hyperplasia in small intestine with an increase in apoptotic (e.g., orange arrowhead) and mitotic (e.g., white arrowhead) figures (higher magnification panel at bottom left). E: constitutive (i.e., prior to tamoxifen treatment) Cre expression levels in various organs in CAGGCreERTM mice using qRT-PCR. Stomach has the highest Cre recombinase expression level except liver (n = 3 animals/group; P < 0.07 for stomach vs. small intestine). *P < 0.05; ***P < 0.001.
by one-way ANOVA followed by either Dunnett or Tukey post hoc multiple comparison correction; n = 3 mice/group), and small intestine had minimal histological phenotype and no increase in DNA damage marker (Fig. 4, A and E). Large intestine and pancreas, neither of which had shown histological phenotype nor DNA damage marker increase, had fourfold less Cre expression than stomach (P < 0.001 for large intestine and P < 0.001 for pancreas). The exception to the trend was liver, which had statistically higher expression (P < 0.05) than stomach but did not show histological damage, although liver had shown increased DNA damage markers. In sum, the data show a trend for higher Cre transgene expression to correlate with increased induction of DNA damage and/or histological injury following tamoxifen injection.

To further explore this interpretation, we analyzed tamoxifen-inducible Cre activity in mice expressing Cre under a different global promoter. R26CreERT mice, which also have broad Cre expression, were analyzed at the same tamoxifen dose and schedule. The Cre genotoxic phenotype was not observed in R26CreERT mouse stomach after tamoxifen injection (Fig. 5A). As would be predicted if genotoxicity correlated positively with level of Cre expression, the constitutive gastric Cre transgene expression level was 2.6-fold less in R26CreERT vs. CAGGCreERTM mice (P < 0.001; n = 3 mice/group). Thus, overall, the Cre genotoxic phenotype correlated with Cre transgene levels both across organs and in the same organ using promoters driving different constitutive levels (see also discussion for why liver might be a partial exception to this pattern).

Stomach has regenerative capacity to recover from Cre genotoxicity-induced injury. Given the extent of the Cre-induced damage to the gastric mucosa, we expected that animals would either not survive long term because of stomach erosion and hemorrhage or that they would develop tumors arising from the regenerative foci. Surprisingly, however, of the seven tamoxifen-treated CAGGCreERTM mice we have followed longer than 2 wk after Cre induction, three survived until we euthanized them several months later. Figure 6 shows the gastric mucosa of such survivors. One animal (taken at 11 wk) showed near complete recovery of the entire mucosa. No hyperplastic regions remained, and nearly all gastric units showed parietal cells with more or less normal zymogenic cell differentiation (Fig. 6A, left). There was some residual unevenness of the luminal surface, and many regions exhibited some degree of irregularity in organization among neighboring gastric units (see Fig. 6B, left, where units normally organized like test tubes in a rack show unit lumens that are not perpendicular to the muscle layers and are of varying lengths in the muscle to gastric surface dimension). The abundant stroma of earlier time points had regressed, as had the inflammation. In another long-term animal, parietal cells and zymogenic cells had returned diffusely, but there was considerably more irregularity in gastric unit length and organization with obvious hyperplasia still present in some regions (Fig. 6, A and B, right). BrdU labeling showed that proliferation rates matched histology. In regions that appeared normal, BrdU incorporation had returned to normal rates; in regions that were histologically hyperplastic and irregular, BrdU immunoreactivity indicated proliferation was still increased (Fig. 6C). Overall, the results suggest that, at least in some mice, the severe mucosal injury induced by Cre recombinase can be repaired, possibly by proliferation and subsequent differentiation of stem cells located in the hyperplastic foci observed at 14 days (e.g., Figs. 1, D and E, and 2C).

Tamoxifen dosage and frequency can be titrated to achieve loxP-specific recombination without nonspecific genotoxicity. Tamoxifen induces Cre recombinase activity, because CreER TM resides in the cytoplasm unless it encounters tamoxifen, which mediates Cre translocation into the nucleus where it can bind genomic DNA. (9) A loxP-flanked gene is permanently deleted in a given cell, as soon as sufficient Cre recombinase activity is present to bind, loop out, and recombine the sequence flanked by loxP sites. After loxP-flanked gene excision or in the absence of any true loxP sites, it follows that increasing Cre concentration in the nucleus would serve only to increase the potential for nonspecific recombination. Indeed, in vitro, Cre activity toward non-loxP sites has been shown to be dose dependent (1, 18). Since tamoxifen-inducible Cre recombinase activity is a critical tool for examination of gene function in organs like the stomach, which exhibits constant homeostatic turnover and largely assumes its adult differentiation state well after birth, it was important to test

Fig. 5. R26CreERT mice have lower gastric Cre recombinase expression than CAGGCreERTM mice and do not show Cre genotoxicity. R26CreERT mice do not show the Cre genotoxic phenotype induced in CAGGCreERTM mice following the same dose and schedule of intraperitoneal tamoxifen injection (5 mg/20 g for 3 days; cf. Fig. 1D). B: R26CreERT stomachs have 2.6-fold lower constitutive Cre recombinase expression level than CAGGCreERTM stomachs. ***P < 0.001.
whether a dose of tamoxifen could be achieved that would not induce nonspecific genotoxicity. To verify the efficiency of DNA recombination in the stomach, we crossed CAGGCre-ERTM mice with R26R reporter mice, where lacZ gene expression is driven by a Rosa26 promoter followed by floxed STOP cassettes. With a dose of 5 mg of tamoxifen per 20 g body wt and 3 days of daily injection (the dose used that generated the phenotypes examined above), DNA recombination efficiency was 100% in the stomach epithelium, both in corpus and antrum, within 3 days (Fig. 7A). To determine whether we could lower the dose of tamoxifen without losing efficiency of loxP-flanked gene excision, we lowered tamoxifen dosage to 1 mg per 20 g body wt. At this dose, we did not observe any Cre genotoxic effect in the stomach. However, DNA recombination efficiency was low. Surface pit cells and zymogenic cells showed almost 100% recombination efficiency, however, the efficiency in parietal cells and neck progenitor cells was less than 5% (Fig. 7B), so we increased frequency, maintaining dosage at 1 mg. When we injected tamoxifen for 7 consecutive days, the recombination efficiency was again 100% (Fig. 7D); however, the stomach showed focal metaplasia on day 7, although other animals in this cohort recovered by day 14 (not shown). We next decreased tamoxifen dosage to 0.75 mg per 20 g body wt with the same frequency. At this schedule, recombination in all epithelial cell types except parietal cells was near 100% and nearly 100% of gastric units showed this pattern (Fig. 7E). With the same dosage and schedule, DNA recombination efficiency was ~90% in antrum where parietal cells are not present (data not shown). In corpus, the LacZ histochemical staining pattern coincided with Cre immunofluorescence staining (Fig. 8). With 5 mg tamoxifen injection per 20 g body wt for 3 days, almost every cell expressed nuclear Cre (Fig. 8A), whereas only the zymogenic cell lineage had nuclear Cre staining with 0.75 mg tamoxifen injection per 20 g body wt for 7 days (Fig. 8D). Wild-type mouse stomach showed only background staining (Fig. 8B), and CAGGCreERTM mouse stomach with vehicle injection showed cytoplasmic Cre staining with occasional weak nuclear staining in zymogenic cells.
These data show that it is possible to obtain specific recombination at floxed alleles without Cre genotoxicity in tamoxifen-inducible Cre lines by titrating tamoxifen dosage and frequency.

Specific deletion of Bmpr1a with titrated tamoxifen dosage and frequency causes antral and pyloric hyperplasia without Cre genotoxicity. We next examined the effects of deletion of a specific floxed gene using the tamoxifen dosing schedule that did not cause Cre-mediated genotoxicity. We injected a cohort of three CAGGCreERTM;Bmpr1aflox/flox mice with 0.75 mg of tamoxifen per 20 g body wt for 7 consecutive days. Two of the mice died (one at 17 days, one at 21 days) after tamoxifen injection. The remaining mouse, who was also unhealthy in appearance, was euthanized at day 21. The three mice were examined by necropsy. All showed prominent hair loss (Fig. 9A). All micrographs at same magnification, scale bar in A.

DISCUSSION

In the experiments in this manuscript, induction of Cre recombinase activity in adult mice, regardless of the presence
of floxed alleles, caused genotoxic tissue damage, consisting of
dramatic atrophy, metaplasia, and foci of hyperplasia. Even
though the promoter driving Cre was expressed in all tissues,
only the stomach showed such injury. Surprisingly, the gastric
mucosa was able to recover to near normalcy several weeks
after these dramatic changes, suggesting that the stomach has
substantial regenerative capacity. We also show here that the
genotoxic damage to the stomach is dose dependent, and Cre
recombinase activity can be titrated to a point at which tissue
damage does not occur but recombination of specific floxed
alleles does. Finally, we induce gene-specific Cre recombinase
to demonstrate the importance of BMP signaling in main-
taining normal proliferation in the gastric antrum.

The potential for Cre genotoxicity in an organ seems to be
positively correlated to the expression level of Cre transgene in
that organ. In CAGGCreER\(^{TM}\) mice, liver, which showed
slightly increased expression of DNA damage markers without
a histological phenotype that following tamoxifen treatment,
had the highest level of constitutive Cre expression among
organs we assayed. Stomach had the second highest level of
constitutive Cre expression, and it showed increased DNA
damage markers along with severe destruction of mucosal
epithelium after tamoxifen. Small intestine expressed twofold
less Cre transgene than stomach although the difference was
not statistically significant, and it showed minimal crypt epithe-
lical hyperplasia and apoptosis without DNA damage marker
increase. Large intestine and pancreas expressed the lowest levels
of Cre and did not show histological change nor increased DNA
damage markers following tamoxifen. Analysis of another uni-
versal Cre expression line, R26\(\text{Cre}^{ER}\) mice, supported the cor-
relation between Cre genotoxicity and Cre expression level.
R26\(\text{Cre}^{ER}\) mice had 2.6-fold less Cre expression level in their
stomachs than the Cre level in CAGGCreER\(^{TM}\) mice stomachs,
and R26\(\text{Cre}^{ER}\) mouse stomach did not show the pattern of
histological damage of CAGGCreER\(^{TM}\) mice at the same dosage
and frequency of tamoxifen injection.

Another factor that could affect Cre genotoxicity is the basal
rate of proliferation in organs. Although more experiments
would be required to directly address this issue, it is interesting
that liver had high constitutive Cre expression and showed
molecular evidence of DNA damage (increased DNA damage
markers) but did not show histological damage, unlike the
stomach. Liver has low basal proliferation that increases in
response to loss of hepatocytes; the gastric epithelium, on the
other hand, is among the few adult tissues that renews constitu-
tively throughout life from a tissue-specific, resident stem
cell. It is known that proliferating cells are more susceptible to
genotoxic stress such as oxidative damage and irradiation than
quiescent cells (19, 39). In sum, the Cre genotoxic phenotype
we observe in stomach seems to depend both on higher
constitutive Cre expression than other organs and the prolifera-
tive characteristic of stomach. Higher levels of Cre expression
result in higher induction of Cre in the nuclei following
tamoxifen treatment. Lowering tamoxifen levels reduces the
amount of nuclear, activated Cre, decreasing genotoxicity,
even in promoters that drive high constitutive levels. However,
our observations do not exclude other possibilities; stomach
epithelial cells might simply be more susceptible to genotoxic
damage than cells in other organs.

We observed SPEM as an early effect of genotoxicity
induced by Cre recombinase. However, it is not clear whether
the metaplasia is the result of genotoxic damage to all the
epithelial cells or to any specific lineage. The loose correlation
of genotoxicity with basal proliferation discussed above sug-
gests that the most important genotoxic effect may be on stem
and progenitor cells. However, the parietal cell is known to be
a master regulator of gastric unit differentiation and prolifera-
tion (8, 13, 21, 36). In its absence, the zymogenic lineage
undergoes SPEM-type metaplasia, and proliferation greatly increases (6, 16, 28, 29). Interestingly, nongenotoxic doses of tamoxifen also happened to be doses that did not induce measurable Cre activity in parietal cells as assessed by lacZ activity in R26R reporter mice (Fig. 7). Thus parietal cell death induced by genotoxicity might also be important in the metaplasia induced by Cre. We do observe apoptotic parietal cells early following induction of Cre activity, but other cell lineages are apoptotic at the same time, and the entire mucosa eventually undergoes complete remodeling that is beyond that seen normally in SPEM, so the effects of Cre in the stomach may involve multiple cell types.

The gastric response to Cre-induced damage that we report here suggests a new model system to study gastric injury. Mice need only express a single copy of the transgene, and three doses of tamoxifen are sufficient to induce dramatic injury in a stereotypic fashion that mimics the pattern of damage induced by Cre. We do observe apoptotic parietal cells early following induction of Cre activity, but other cell lineages are apoptotic at the same time, and the entire mucosa eventually undergoes complete remodeling that is beyond that seen normally in SPEM, so the effects of Cre in the stomach may involve multiple cell types.

That the damage is so extensive but is reversible may be a useful aspect of Cre recombinase-induced mucosal injury. Despite near complete atrophy of the gastric mucosa, three of seven mice survived long term following this injury, and, in those mice, eventually, the mucosa repaired itself. What are the mechanisms of this repair? Some of these foci may derive from stem cells resistant to high-dose Cre that then proliferate to replace the entire gastric mucosa. Given that by day 14 there were no mature parietal or zymogenic cells present in any animals anywhere in the stomach, this indicates tremendous proliferative and regenerative potential of the gastric epithelial stem cell. We are currently examining the cellular and molecular mechanisms at play in the ability of the stomach to regenerate and differentiate even in the adult, because these mechanisms may be key in understanding how to repair human gastric mucosa in patients who have suffered metaplasia and atrophy in the face of long-term Helicobacter infection.

One interesting observation during Cre genotoxic damage in stomach was that epithelial damage was much more severe in gastric corpus than in antrum. The corpus epithelium was eventually atrophied to a single cell layer overlying an edematous stroma by 14 days after tamoxifen injection into CAGGCreER™ mice. The antrum (or distal stomach, since definitive identification of the original antrum in this extensive pattern of injury was not possible), on the other hand, had more remaining epithelial cells.
and multiple regenerating foci. Recently, it has been reported that stem cells in gastric antrum express the same marker, Lgr5, as small intestine and colon, whereas the gastric corpus stem cell does not label specifically with Lgr5 (2). So gastric antral epithelium might also have different responses to injury.

Our analysis of BMP signaling again highlighted antrum-corpus differences in regeneration. When BMP signaling was disrupted at nongenotoxic levels of Cre recombinase activity, we observed alopecia and profound hyperplasia in the gastric antrum and pylorus, but corpus homeostasis was unaffected. There have been several previous reports on BMP signaling in the antrum. Downregulation of BMP signaling by transgenic expression of the antagonist Noggin in the presence of transgenically induced prostaglandin E2 caused antral hamartomatous polyps resembling those of juvenile polyposis (30). Cre recombinase-induced deletion of Bmpr1a by use of an Mxi-Cre system caused hyperplasia and polyps in the antrum more exuberantly even than what we report in the present study (3). The Mxi promoter, like the modified chicken actin we use here, is globally expressed, although induction is not by tamoxifen but by the proinflammatory injection of poly-IC, which could confound interpretation by inducing both gene deletion and inflammation. Multiple previous studies have shown key roles for BMP2, BMP4, and/or BMP7 signaling (the ligands for BMPRIA) in suppressing gastric proliferation in gastric neoplasia in mice and humans (4, 14, 41, 42). Finally, BMP2, 4, and 7 signaling plays a key role in chicken stomach gastrulation (25).

Given the absence of phenotype we observe in the corpus, despite deletion of BMPRIA there, the results suggest that this branch of BMP signaling is dispensable for maintaining homeostasis there. Isolated parietal cells, which reside exclusively in the corpus, respond to BMP4 in vitro by increasing expression of H+/K+-ATPase, which parietal cells use to acidify the gastric lumen; however, those results do not necessarily indicate a requirement for BMP signaling during normal homeostasis of the stomach corpus in vivo (26). We are currently interested in deciphering the mechanisms of the antral BMP signaling. Is the receptor expressed in the epithelium, with BMP ligands derived from the mesenchyme as reported in chicken and during inflammation (4, 25)?

Together, our results show hypersensitivity of the adult gastric mucosa to genotoxic injury as caused by induction of Cre recombinase under regulation of a globally expressed promoter. The stomach also shows remarkable ability to recover after several months from this damage, but investigators working with inducible Cre in the stomach should exercise caution in selection of doses to use as well as selecting appropriate controls (e.g., examining mice with activated Cre but wild-type alleles) when interpreting results in inducible Cre genetic studies. On the other hand, given that Cre-induced gastric injury is rapid and simple, it might be useful as a model to study the long-term regenerative capacity of the adult gastric stem cell for regeneration as well as the mechanisms the stem cells use to expand and reestablish the normal adult gastric epithelial lineages.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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Together, our results show hypersensitivity of the adult gastric mucosa to genotoxic injury as caused by induction of Cre recombinase under regulation of a globally expressed promoter. The stomach also shows remarkable ability to recover after several months from this damage, but investigators working with inducible Cre in the stomach should exercise caution in selection of doses to use as well as selecting appropriate controls (e.g., examining mice with activated Cre but wild-type alleles) when interpreting results in inducible Cre genetic studies. On the other hand, given that Cre-induced gastric injury is rapid and simple, it might be useful as a model to study the long-term regenerative capacity of the adult gastric stem cell for regeneration as well as the mechanisms the stem cells use to expand and reestablish the normal adult gastric epithelial lineages.
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