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ORIGINAL PAPER

Bmi1 marks gastric stem cells located in the isthmus in mice

Takuto Yoshioka[1], Akihisa Fukuda[1], Osamu Araki[1], Satoshi Ogawa[1], Yuta Hanyu[1], Yoshihide Matsumoto[1], Yuichi Yamaga[1], Yuki Nakanishi[1], Kenji Kawada[2], Yoshiharu Sakai[2], Tsutomu Chiba[1,3] and Hiroshi Seno[1]*

[1] *Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, Kyoto, Japan*
[2] *Department of Surgery, Kyoto University Graduate School of Medicine, Kyoto, Japan*
[3] *Kansai Electric Power Hospital, Osaka, Japan*

**Correspondence to: H Seno, Department of Gastroenterology and Hepatology, Kyoto University Graduate School of Medicine, 54 Shogoin-Kawahara-cho, Sakyo-ku, Kyoto 606-8507, Japan. E-mail: seno@kuhp.kyoto-u.ac.jp*

Abstract

In the mammalian stomach, the isthmus has been considered as a stem cell zone. However, various locations and proliferative activities of gastric stem cells have been reported. We focused here on the stem cell marker Bmi1, a polycomb group protein, aiming to elucidate the characteristics of Bmi1-expressing cells in the stomach and to examine their stem cell potential. We investigated the Bmi1-expressing cell lineage in *Bmi1-CreERT; Rosa26-YFP, LacZ* or *Rosa26-Confetti* mice. We examined the *in vivo* and *ex vivo* effects of Bmi1-expressing cell ablation by using *Bmi1-CreERT; Rosa26-iDTR* mice. The Bmi1 lineage was also traced during regeneration after high-dose tamoxifen-, irradiation- and acetic acid-induced mucosal injuries. In the lineage-tracing experiments using low-dose tamoxifen, Bmi1-expressing cells in the isthmus of the gastric antrum and corpus provided progeny bidirectionally, towards both the luminal and basal sides over 6 months. In gastric organoids, Bmi1-expressing cells also provided progeny. Ablation of Bmi1-expressing cells resulted in impaired gastric epithelium in both mouse stomach and organoids. After high-dose tamoxifen-induced gastric mucosal injury, Bmi1-expressing cell lineages expanded and fully occupied all gastric glands of the antrum and the corpus within 7 days after tamoxifen injection. After irradiation- and acetic acid-induced gastric mucosal injuries, Bmi1-expressing cells also contributed to regeneration. In conclusion, Bmi1 is a gastric stem cell marker expressed in the isthmus of the antrum and corpus. Bmi1-expressing cells have stem cell potentials, both under physiological conditions and during regeneration after gastric mucosal injuries.
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Introduction

Stem cells harbor the ability to self-renew and to produce differentiated progeny. Tissue stem cells maintain the homeostasis of mammalian tissues [1]. In stem cell research, the intestines are the most surveyed digestive organs, due to their rapid turnover rate [2]. In contrast, only a few studies have reported the existence and biological significance of gastric stem cells using gastric stem cell 'markers' [3–8]. Challenges to stem cell research in the stomach include the slow turnover rate (3–7 days in pit cells and several months in chief and parietal cells) [9] and the histological complexity of this organ. The antrum and corpus of the mammalian stomach have different histological structures. The antrum consists of units, including a long pit, an isthmus (where proliferative cells are located), and a base. The corpus consists of units, including a short pit, an isthmus one-third of the distance from the luminal side, a long neck and a base. The isthmus has long been considered to be a stem cell zone, based on the existence of undifferentiated cells [10]; however, several studies using lineage-tracing methods have shown that gastric stem cells exist in the lower part of the gastric unit. Leucine-rich repeat-containing G-protein coupled receptor 5 (Lgr5), a well-characterized intestinal stem cell marker, marks gastric antral stem cells located in the base [3]. Another report identified the novel stem cell marker cholecystokinin 2 receptor (Cckbr), which marks antral stem cells located in the +4 position of gastric units [6]. TNF receptor superfamily member 19 (Troy) was reported to mark reserve stem cells located in the base after 5-fluorouracil (5-FU)-induced injury [5]. On the other hand, the basic helix–loop–helix transcription factor, Mist1, a differentiated chief cell marker, was reported to mark stem cells located in the isthmus [7]. However, Mist1-positive stem cells rarely express proliferative markers. Another stem cell marker, Runx1 enhancer element (eR1), was reported to be expressed in the rapidly proliferating stem cells in the isthmus of the corpus [8]. More recently, using *Lgr5-2A-CreERT* mice, Lgr5-positive chief cells in the base were reported to act as reserve stem cells after high-dose tamoxifen-induced

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injury [9]. These reports have shown different locations and different proliferating ratios of gastric stem cells, and the dosage of tamoxifen differed between studies, which may influence gastric homeostasis because of damage to parietal cells [10].

Bmi1 is a member of the polycomb-repressing complex 1, which plays an essential role in maintaining chromatin silencing [11], and is involved in the self-renewal of neuronal, tongue, hematopoietic and leukemic cells [12–15]. In the small intestine, Bmi1-positive cells reside in the +4 position above the intestinal crypt base. They are thought to serve as a reserve stem cell pool in response to mucosal injury and as a source for replenishment of Lgr5-positive rapid cycling intestinal stem cells under non-pathological conditions [16]. Here we focused on Bmi1 and examined the potential of Bmi1-expressing cells as gastric stem cells.

Materials and methods

Mice

Bmi1-CreERT (#010531, generated by M Capecchi), *Lgr5-EGFP-CreERT* (#008875), *Rosa26-LacZ* (#003474), *Rosa26-YFP* (#006148), *Rosa26-Confetti* (#017492) and *Rosa26-iDTR* (JAX #007900) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). *eRx1-CreERT* mice were gifted by M Osato and Y Ito from the Cancer Science Institute of Singapore [8]. For induction of Cre-mediated recombination, we used low and high dosage tamoxifen protocols. For low-dose tamoxifen treatment, 8-week-old mice were injected i.p. with 0.1 mg/g body weight. For high-dose tamoxifen treatment, 8-week-old and >30-week-old mice were injected with 4 mg/mouse tamoxifen. For 5-FU treatment, adult mice were injected with 150 mg/kg 5-FU (F6627; Sigma-Aldrich, St Louis, MO, USA) i.p. 1 and 2 days after injection of 0.1 mg/g tamoxifen. Diphtheria toxin (DT) was provided by K Kohno from the Nara Institute of Science and Technology (Ikoma, Nara, Japan). For ablation of Bmi1-expressing cells, adult mice were injected i.p. with 100 ng DT every 8 h for 6 consecutive days and 2 mg/mouse tamoxifen every 24 h for 5 consecutive days. The first tamoxifen was administered 24 h after the initial DT injection. Mice were sacrificed 2 h after the last injection of DT. For quantification of Bmi1-positive cells, all gastric glands were examined in three individual sections selected randomly. The locations of all Bmi1-positive cells were mapped on graphs; the distribution ratio was plotted on the *x*-axis and the position from the base on the *y*-axis. All experiments were approved by the animal research committee of Kyoto University and performed in accordance with Japanese government regulations.

Gastric ulcer induction

Adult *Bmi1-CreERT; Rosa26-LacZ* mice were injected i.p. with 3.3% trichloroacetaldehyde hydrate for general anesthesia and a small abdominal incision was made. An aliquot of 5 µl 100% acetic acid was applied on the serosa of the gastric antrum and corpus for 30 s. After washing with PBS, the peritoneum and skin were sutured with silk thread.

Immunostaining

Mouse tissues were fixed with 4% buffered paraformaldehyde solution, paraffin embedded and sectioned. For immunohistochemistry, sections were incubated with primary antibody overnight, then with biotinylated secondary antibody for 1 h; immunoperoxidase labeling was performed using a Vectastain ABC kit (Vector Laboratories, Burlingame, CA, USA) and sections were colored with diaminobenzidine substrate (Dako, Santa Clara, CA, USA) and counterstained with hematoxylin. For immunofluorescence, sections were incubated with primary antibody overnight, incubated with fluorescent dye-conjugated secondary antibody (Invitrogen, Carlsbad, CA, USA) for 1 h and stained with Hoechst dye. Primary antibodies were obtained from the indicated suppliers: goat anti-GFP (×500; ab6673, Abcam, Cambridge, MA, USA), rat anti-Ki67 (×100; M7249, Dako), anti-Muc5AC (×100; ab3649, Abcam), rabbit anti-chromogranin A (×100; ab15160, Abcam), rabbit anti-Dclk1 (×200; ab31704, Abcam), mouse anti-spasmolytic polypeptide IgM (×100; ab49536, Abcam), mouse anti-proton pump (×1; D031-3H, Medical & Biological Laboratories, Nagoya, Aichi, Japan), rabbit anti-pepsinogen1 (×100; 17330-1-AP, Proteintech, Rosemont, IL, USA), sheep anti-pepsinogen2 (×10000; ab9013, Abcam), rabbit anti-human Bmi1 (×400; #6964S, Cell Signaling Technology, Danvers, MA, USA), goat anti-murine Bmi1 (×300; ab115251, Abcam) and rabbit anti-cleaved caspase3 (×100; #9664, Cell Signaling Technology). TUNEL staining was performed using the In Situ Cell Death Detection Kit (Roche, Mannheim, Germany). Isotype controls were used as negative controls.

X-gal staining

Freshly isolated mouse stomach was incubated in ice-cold fixative solution, which contained 4% paraformaldehyde, 0.2% glutaraldehyde, 5 mM EGTA, 2 mM $MgCl_2$ and 0.02% NP40, for 1 h. After washing in PBS, tissues were incubated with LacZ substrate, which contained 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% NP40, 0.1% sodium deoxycholate and 1 mg/ml X-gal. After washing with PBS, tissues were fixed in 4% paraformaldehyde in PBS. Paraformaldehyde was removed with 70% ethanol. Tissues were embedded in paraffin, sectioned and stained with nuclear fast red.

Human subjects

Surgically resected specimens were obtained from human gastric cancer patients at Kyoto University

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Hospital. Samples were processed for immunohistochemistry after fixing in 10% formalin. Analyses of human subjects were approved by the ethical committee of Kyoto University Hospital and informed consent was obtained from all patients.

Statistical analysis

All values are presented as mean ± SEM. Student's *t*-test and Welch's *t*-test were used for statistical analysis. *P* values < 0.05 were considered to be significant.

Results

Bmi1-expressing cells are mainly located in the isthmus epithelium in humans and mice, and are unique from other differentiated or stem cells in mice

To survey the location of BMI1-expressing cells in the gastric unit we immunostained BMI1 protein in normal human gastric epithelium. The majority of BMI1-expressing cells were present in the isthmus of the gastric antrum and corpus (64.5 and 57.6%, respectively) (Figure 1A). Next we used *Bmi1-CreERT; Rosa26-YFP or LacZ* mice because immunohistochemistry for Bmi1 showed an unclear signal in mice. After a single administration of low-dose tamoxifen (0.1 mg/g body weight), which has no metaplastic influence on gastric epithelium [10], to 8-week-old *Bmi1-CreERT; Rosa26-YFP or LacZ* mice, immunohistochemistry for GFP and differentiation markers was performed in the gastric epithelium. The induction rate of this construct was low, with only 2.84% positive cells on average, and YFP- or X-gal-positive Bmi1-expressing cells were scattered in the gastric epithelium. We simultaneously confirmed that Bmi1 and YFP were co-expressed in the same cell in *Bmi1-CreERT; Rosa26-YFP* mice at 1 day after tamoxifen injection (see supplementary material, Figure S1A). YFP- or X-gal-positive Bmi1-expressing cells mainly existed in the isthmus of the gastric antrum and corpus (68.7 and 57.5%, respectively) on day 1 after tamoxifen injection (Figure 1B). The frequency of a single Bmi1-expressing cell per gastric gland was 0.49% on average. Bmi1-expressing cells did not co-express markers for differentiated cells, including Muc5AC-positive pit cells, chromogranin A (ChgA)-positive neuroendocrine cells, Dclk1-positive tuft cells, spasmolytic polypeptide (Tff2)-positive mucous neck cells and pepsinogen 1 (Pga1)-positive chief cells in the gastric epithelium (Figure 1C). We also examined the colocalization of Bmi1 and other stem cell markers, Lgr5 and eR1. First, we performed immunohistochemistry for Bmi1 and GFP in *Lgr5-EGFP-CreERT* mice and detected no colocalization of Bmi1 and GFP. Second, we performed immunohistochemistry for Bmi1 and GFP in 8-week-old *eR1-CreERT; Rosa26-YFP* mice at 1 day after low-dose tamoxifen injection and again detected no colocalization of Bmi1 and GFP (Figure 1D). These data suggest that Bmi1-expressing cells are unique from other differentiated gastric cells and may be different populations from Lgr5- and eR1-expressing cells.

Bmi1-expressing cells in the isthmus have the potential of normal stem cells in mouse gastric antrum and corpus

To survey the stemness of Bmi1-expressing cells, we performed lineage tracing using 8-week-old *Bmi1-CreERT; Rosa26-YFP or LacZ* mice after a single injection of low-dose tamoxifen. Serial observation showed that YFP- and X-gal-positive cells continuously provided progeny from the isthmus to pit and base in the gastric antrum and corpus over 1 week (Figure 2A) and up to 6 months (Figure 2B), respectively. X-gal-positive gastric glands were observed in the gastric antrum and corpus on day 7 (3.69 and 1.98%, respectively) (Figure 2C) and at 6 months (1.35 and 1.37%, respectively). Importantly, we observed the expression of all the gastric differentiated cell markers within X-gal-positive gastric epithelial cells of *Bmi1-CreERT; Rosa26-LacZ* mice 6 months after low-dose tamoxifen injection (Figure 2D). These data support the notion that Bmi1-expressing cells can act as gastric stem cells that can give rise to all types of gastric epithelial cell. To clarify in which direction Bmi1-expressing cells provide progeny, we next generated *Bmi1-CreERT; Rosa26-Confetti* mice. A dose of 4 mg tamoxifen was administered to 38-week-old mice at 30 weeks after injecting 2 mg tamoxifen for 5 consecutive days, and endogenous fluorescent signals in frozen sections of the gastric epithelium were determined 1.5 days later. These treatments did not cause gastric epithelial damage, including parietal cell loss. Bmi1 lineage-traced cells that expressed cytoplasmic YFP or nuclear GFP expanded with a single isthmus cell that expressed cytoplasmic RFP in the antrum and the corpus (Figure 2E). These data suggest that a Bmi1-expressing cell in the isthmus (RFP-positive) divided and provided progeny bidirectionally towards both the luminal and basal sides. Taken together, Bmi1-expressing cells in the isthmus have stem cell potential in mouse gastric antrum and corpus under physiological conditions.

Bmi1-expressing cells in the isthmus were highly proliferative and susceptible to 5-FU treatment

To survey the proliferative potential of Bmi1-expressing cells in the gastric epithelium, we performed immunohistochemistry for GFP and Ki67 in *Bmi1-CreERT; Rosa26-YFP* mice 1 day after low-dose tamoxifen injection. Bmi1-expressing cells in the isthmus were positive for Ki67 in proportions of 71.0 and 57.4% in the antrum and corpus, respectively (Figure 3A,B), which were higher than those in Lgr5-expressing cells (29 and 35% in the antrum and corpus, respectively) in previous reports [3,9]. We then examined whether the Bmi1-expressing cell lineages were inhibited by 5-FU,

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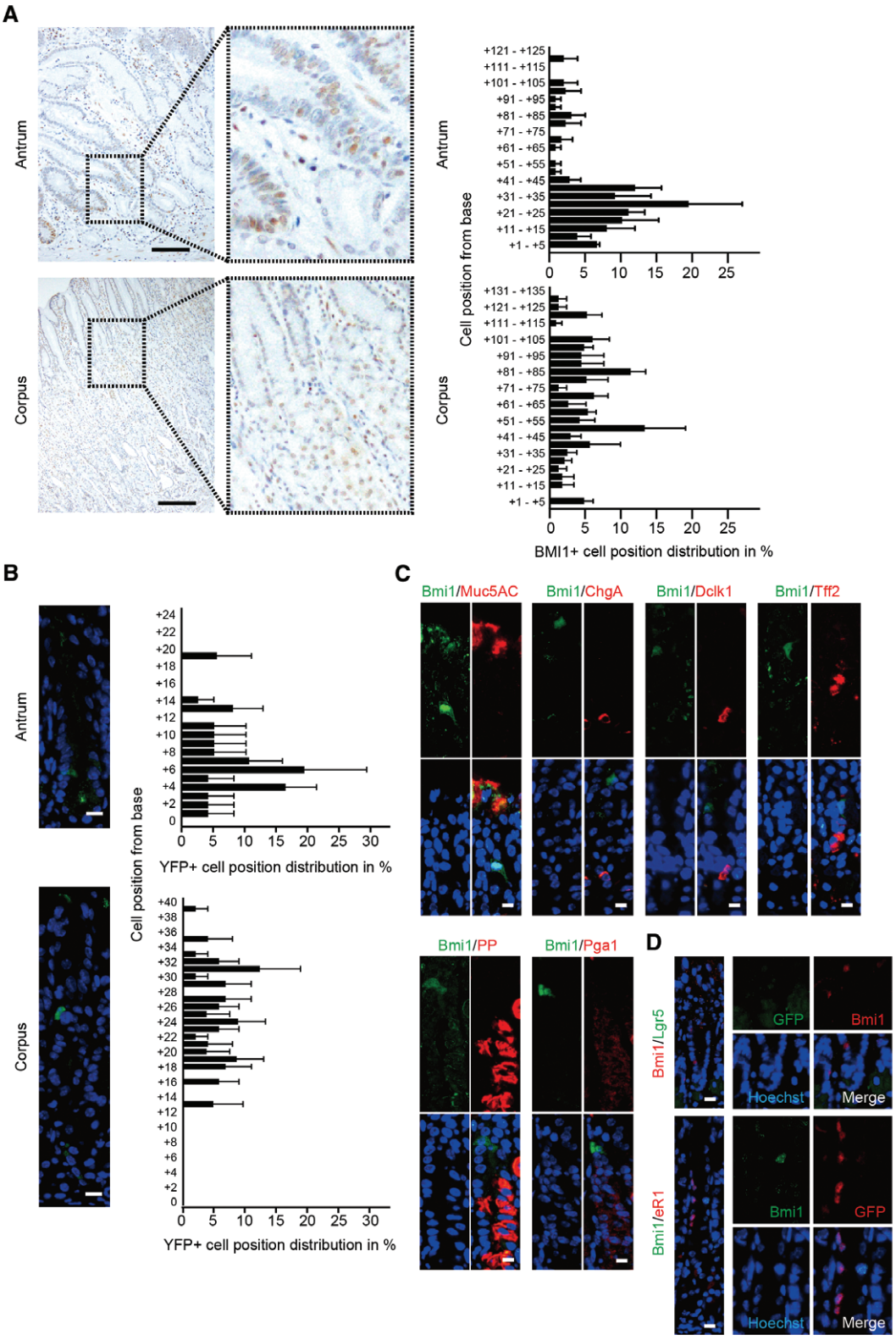

Figure 1. Bmi1-expressing cells were mainly located in the isthmus of the stomach and were unique from other differentiated or stem cells. (A) Immunohistochemistry for BMI1 in the normal human gastric epithelium. The location of BMI1-positive cells and the distribution ratio in the human gastric epithelium were plotted ($n = 3$). Scale bars: 50 μm. (B) Immunohistochemistry for GFP in 8-week-old *Bmi1-CreERT; Rosa26-YFP* mice 1 day after 0.1 mg/g body weight tamoxifen injection. Bar graphs show the distribution of Bmi1+/YFP+ cells in the gastric epithelium ($n = 3$). Scale bars: 10 μm. (C) Immunofluorescent double staining for GFP and gastric differentiated cell markers: Muc5AC, chromogranin A (ChgA), Dclk1, Tff2, proton pump (PP) and pepsinogen1 (Pga1) in 8-week-old *Bmi1-CreERT; Rosa26-YFP* mice 1 day after 0.1 mg/g body weight tamoxifen injection. Scale bars: 10 μm. (D) Immunofluorescent double staining for Bmi1 and GFP on day 1 after 0.1 mg/g body weight tamoxifen injection in *Lgr5-EGFP-CreERT* mice and *eR1-CreERT; Rosa26-YFP* mice. Scale bars: 10 μm.

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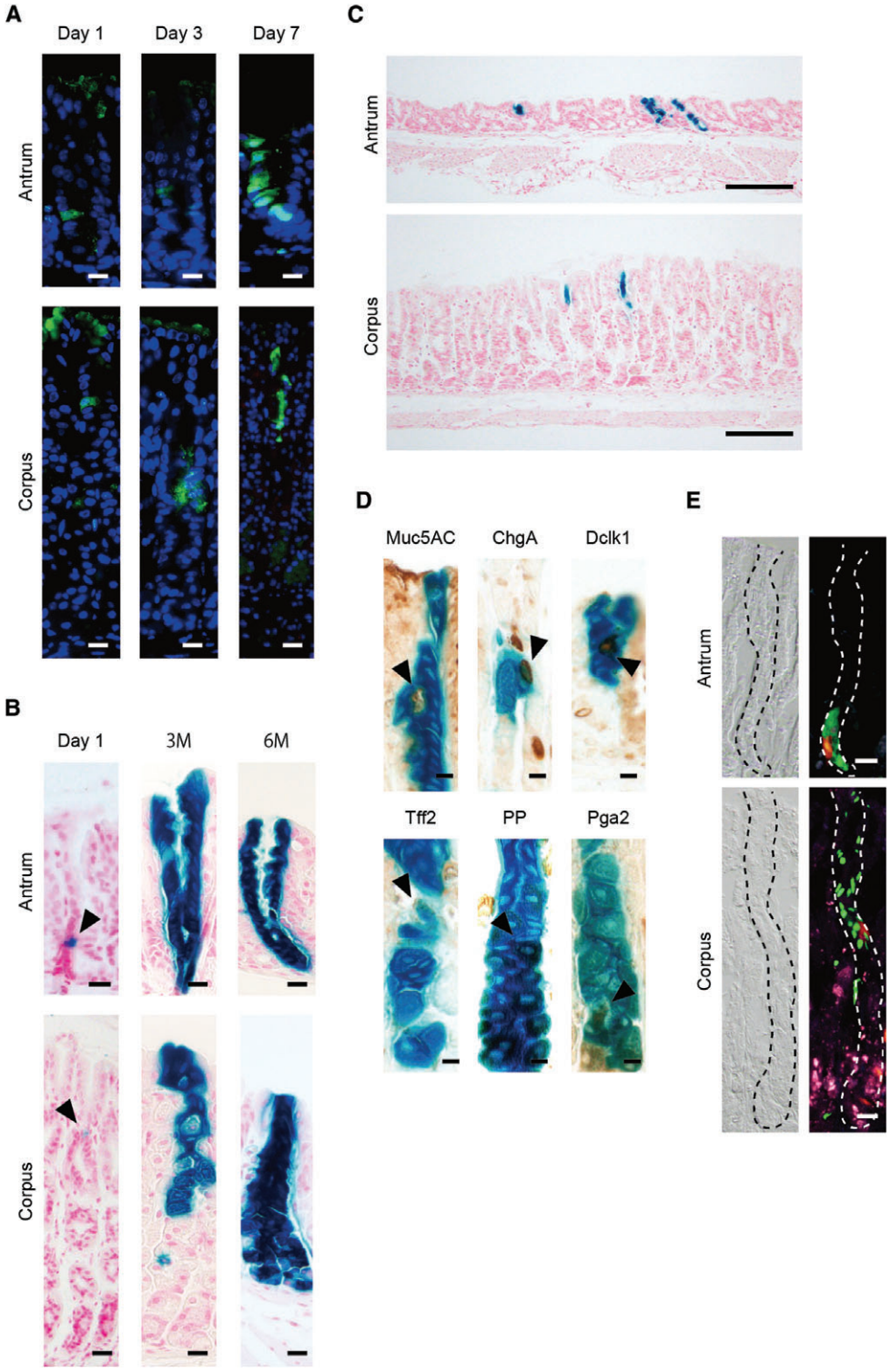

Figure 2. Bmi1-expressing cells provided progeny of all gastric lineages bidirectionally towards the luminal and the basal sides after low-dose tamoxifen injection over 6 months. (A) Immunohistochemistry for GFP in the normal mouse gastric epithelium. Lineage tracing on days 1, 3 and 7 after 0.1 mg/g body weight tamoxifen injection to 8-week-old *Bmi1-CreERT; Rosa26-YFP* mice. Scale bars: 10 μm. (B) Lineage tracing on day 1, 3 months and 6 months after 0.1 mg/g body weight tamoxifen injection to 8-week-old *Bmi1-CreERT; Rosa26-LacZ* mice. Arrowheads show LacZ-positive cells on day 1. Scale bars: 10 μm. (C) LacZ staining of the gastric epithelium in *Bmi1-CreERT; Rosa26-LacZ* mice 1 week after 0.1 mg/g body weight tamoxifen injection. Scale bars: 50 μm. (D) Immunohistochemistry for gastric differentiation markers within LacZ-stained gastric epithelium, 6 months after 0.1 mg/g body weight tamoxifen injection to 8-week-old *Bmi1-CreERT; Rosa26-LacZ* mice. Arrowheads show DAB+ cells within LacZ-labeled gastric glands. Scale bars: 10 μm. (E) Gastric epithelium of *Bmi1-CreERT; Rosa26-Confetti* mice. A dose of 4 mg/mouse tamoxifen was injected 30 weeks after 2 mg/mouse tamoxifen injection for 5 consecutive days. The antrum showed a single Bmi1-expressing cell colored by cytoplasmic RFP and progeny cells with cytoplasmic YFP. The corpus showed a single Bmi1-expressing cell with cytoplasmic RFP and progeny cells colored by nuclear GFP. Scale bars: 10 μm.

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which causes apoptosis of isthmus cells in the gastric epithelium [5]. Low-dose tamoxifen was injected into *Bmi1-CreERT; Rosa26-LacZ* mice and a dose of 5-FU (150 mg/kg) was injected on days 1 and 2 after tamoxifen injection. One week after the tamoxifen injection, no apparent Bmi1 lineages were observed (Figure 3C). Thus, rapidly proliferating Bmi1-expressing cells in the isthmus were sensitive to 5-FU administration.

High-dose tamoxifen induced rapid and efficient production of progeny cells from Bmi1-expressing cells in the isthmus

To investigate the role of Bmi1-expressing cells during regeneration after mucosal injury, we injected *Bmi1-CreERT; Rosa26-LacZ* mice with 4 mg/body tamoxifen, a dose that causes parietal cell loss [10]. We performed immunohistochemistry for proton pump, cleaved caspase 3 and Tff2 on day 3 and X-gal staining on days 1, 3, 7 and 457 after tamoxifen administration. In the corpus epithelium 3 days after high-dose tamoxifen administration, parietal cell loss and apoptosis in the neck lesion were observed, and X-gal- and Tff2-positive spasmolytic polypeptide-expressing metaplasia (SPEM) cells emerged in the neck lesion (Figure 4A). Gastric glands were fully traced by X-gal-positive cells on day 7 in the antrum and corpus, and lineage-traced glands lasted over 1 year (Figure 4B). Frequencies of X-gal-positive gastric glands on day 7 were 12.6 and 20.7% in the antrum and corpus, respectively (Figure 4C). To characterize the Bmi1-expressing cells during regeneration, we performed immunohistochemistry for GFP and Ki67 or differentiated markers in 8-week-old *Bmi1-CreERT; Rosa26-YFP* mice 1 day after high-dose tamoxifen injection. Bmi1-expressing cells mainly existed in the isthmus of the antrum and corpus (55.8 and 55.5%, respectively) (see supplementary material, Figure S1B) and had high ratios of colocalization with Ki67 comparable with the physiological condition (60.0 and 52.8% in the antrum and corpus, respectively) (see supplementary material, Figure S1C,D). Differentiation markers were not colocalized with Bmi1/GFP-positive cells (see supplementary material, Figure S1E). To examine whether Bmi1-expressing cells provide all gastric lineages after high-dose tamoxifen administration, double staining for GFP and differentiation markers was performed in *Bmi1-CreERT; Rosa26-YFP* mice 24 weeks after 4 mg/mouse tamoxifen injection. All gastric differentiation markers were expressed in Bmi1 lineage cells in gastric epithelium (see supplementary material, Figure S2A). These data suggest that parietal cell damage by high-dose tamoxifen caused rapid and efficient production of progeny cells from Bmi1-expressing cells in the isthmus.

Bmi1 marked stem cells in injured gastric epithelium

We next asked if Bmi1-expressing cells exhibit stemness during regeneration after gastric mucosal injury. Whole-body irradiation (8 Gy) was administered to wild-type or *Bmi1-CreERT; Rosa26-LacZ* mice. To examine apoptosis and proliferation of gastric epithelial cells after irradiation, TUNEL staining and immunohistochemistry for Ki67 were performed in wild-type mice. TUNEL-positive apoptotic cells were observed in the isthmus 1 day after irradiation, whereas they were observed in the pit without irradiation (Figure 5A). Ki67-positive cells were observed in the isthmus 1 and 2 days after irradiation, and their frequency increased 2 days after irradiation (1.93 and 4.02 Ki67-positive cells per gland, respectively) (Figure 5B). Next, to perform lineage tracing after irradiation, we injected 0.1 mg/g body weight tamoxifen 1 day after irradiation to 8-week-old *Bmi1-CreERT; Rosa26-LacZ* mice. LacZ-positive, Bmi1-expressing cells were mainly located in the isthmus on day 3 after the tamoxifen injection (57.9 and 55.6% in the antrum and corpus, respectively), and they provided LacZ-positive progeny both in the gastric antrum and corpus on day 7 after the injection, with frequencies of 1.79 and 1.08%, respectively (Figure 5C). Thus, proliferating cells emerged in the isthmus and Bmi1-expressing cells rapidly provided progeny in the gastric epithelium after the irradiation. Next, we generated a mouse gastric ulcer model with minor modification of a previous protocol [17]. Acetic acid (100%) was applied to the anterior serous surface of the gastric antrum and corpus of *Bmi1-CreERT; Rosa26-LacZ* mice 2 days after tamoxifen injection. On the 14th day, when the acetic acid-induced gastric ulcer was healed, X-gal staining was performed. LacZ-positive traced cells were detected and more frequently located beside the ulcer beds than in normal mucosa far from the beds (Figure 5D,E). To survey whether Bmi1-expressing cells provided Tff2-positive metaplastic cells during regeneration of gastric ulcer, immunohistochemistry for Tff2 was performed 14 days after tamoxifen injection and gastric ulcer construction. Tff2 was expressed in the LacZ-positive traced cells besides the ulcer bed (see supplementary material, Figure S2B). These data indicate that Bmi1-expressing cells play a substantial role in regeneration from gastric ulcer formation.

Bmi1-expressing cells were required for gastric epithelial homeostasis

To further confirm the stemness of Bmi1-expressing cells, we examined the requirement for Bmi1-expressing cells to maintain the homeostasis of the gastric epithelium. We generated *Bmi1-CreERT; Rosa26-iDTR* mice and injected DT (100 ng DT every 8 h for 6 days) and tamoxifen (2 mg tamoxifen for 5 days), which resulted in selective killing of Bmi1-expressing stem/progenitor cells. The DT- and tamoxifen-injected *Bmi1-CreERT; Rosa26-LacZ* mice did not show any injured gastric epithelium. However, in contrast, DT- and tamoxifen-treated *Bmi1-CreERT; Rosa26-iDTR* mice displayed injured gastric epithelium, especially around the isthmus region (Figure 6A). The percentages

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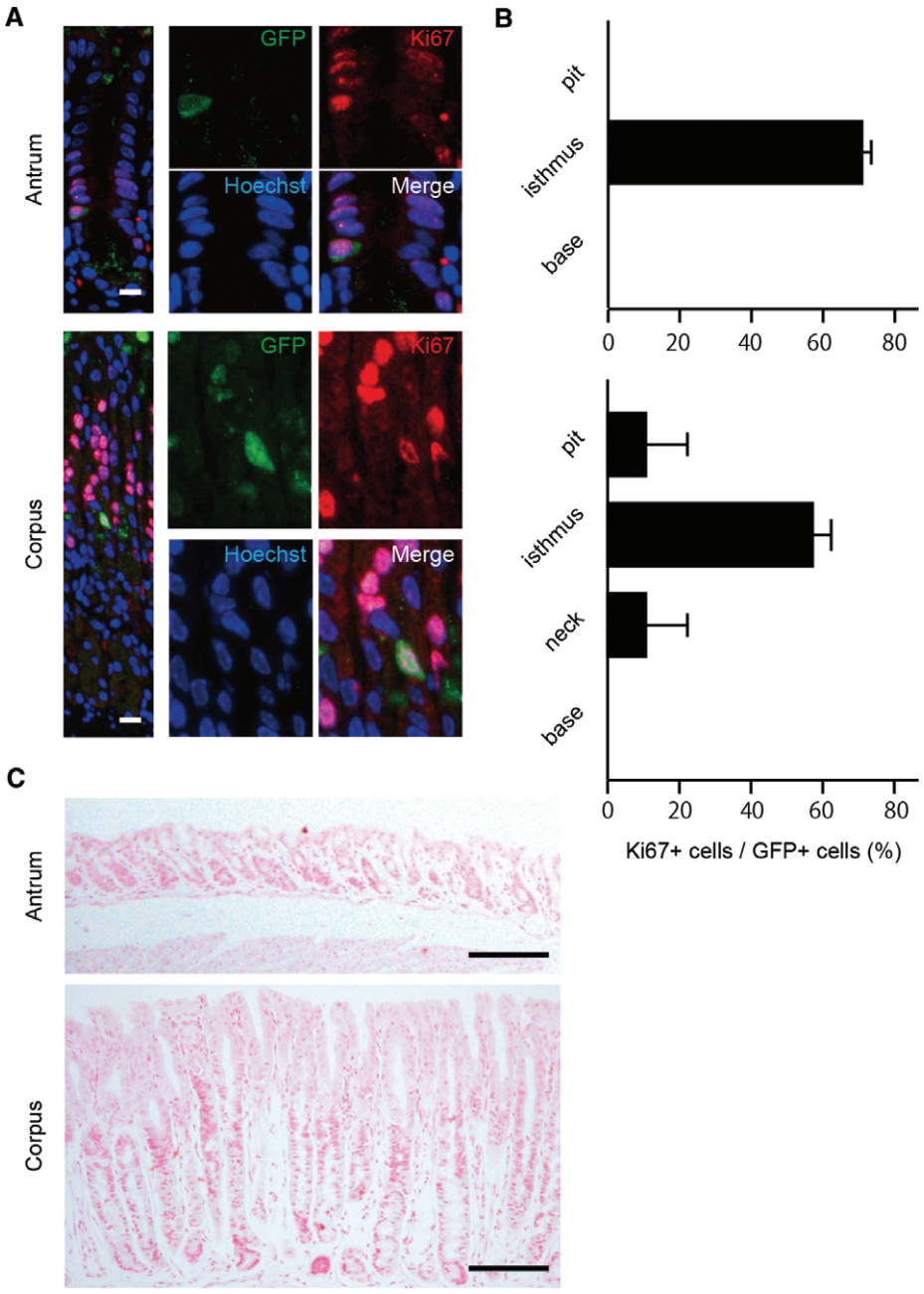

Figure 3. Bmi1-expressing cells in the isthmus were highly proliferative and susceptible to 5-FU treatment. (A) Immunofluorescent double staining for GFP and Ki67 in *Bmi1-CreERT; Rosa26-YFP* mice 1 day after 0.1 mg/g body weight tamoxifen injection. Scale bars: 10 μm. (B) Percentage of Ki67-positive cells in Bmi1-expressing cells in *Bmi1-CreERT; Rosa26-YFP* mice 1 day after 0.1 mg/g body weight tamoxifen injection ($n = 3$). (C) LacZ staining of the gastric epithelium in adult *Bmi1-CreERT; Rosa26-LacZ* mice after the injection of 0.1 mg/g body weight tamoxifen on the first day and 150 mg/kg 5-FU on the second and third days. Scale bars: 50 μm.

of injured glands were 11.2 and 15.0% in the antrum and corpus, respectively. One week after the last DT administration, the gastric epithelium was restored to normal in DT- and tamoxifen-treated *Bmi1-CreERT; Rosa26-iDTR* mice (Figure 6B). To confirm an abundance of LacZ-labeled gastric glands in DT- and tamoxifen-treated mice, lineage tracing was performed in *Bmi1-CreERT; Rosa26-LacZ* mice, which harbor no *Rosa26-iDTR* allele, with the same protocol, showing a similar abundance of LacZ-labeled gastric glands, 10.1 and 11.3% in the antrum and corpus, respectively (see supplementary material, Figure S2C). These data represent the higher induction ratio with 5 days of consecutive injection of 2 mg of tamoxifen than with 0.1 mg/g tamoxifen. Therefore, Bmi1-expressing cells appear to be required for the homeostasis of the gastric epithelium both in the antrum and the corpus.

Bmi1-expressing cells provided progeny in gastric organoids

To confirm the stemness of Bmi1-expressing cells *ex vivo*, we prepared gastric organoids from the gastric antrum and corpus epithelium of *Bmi1-CreERT;*

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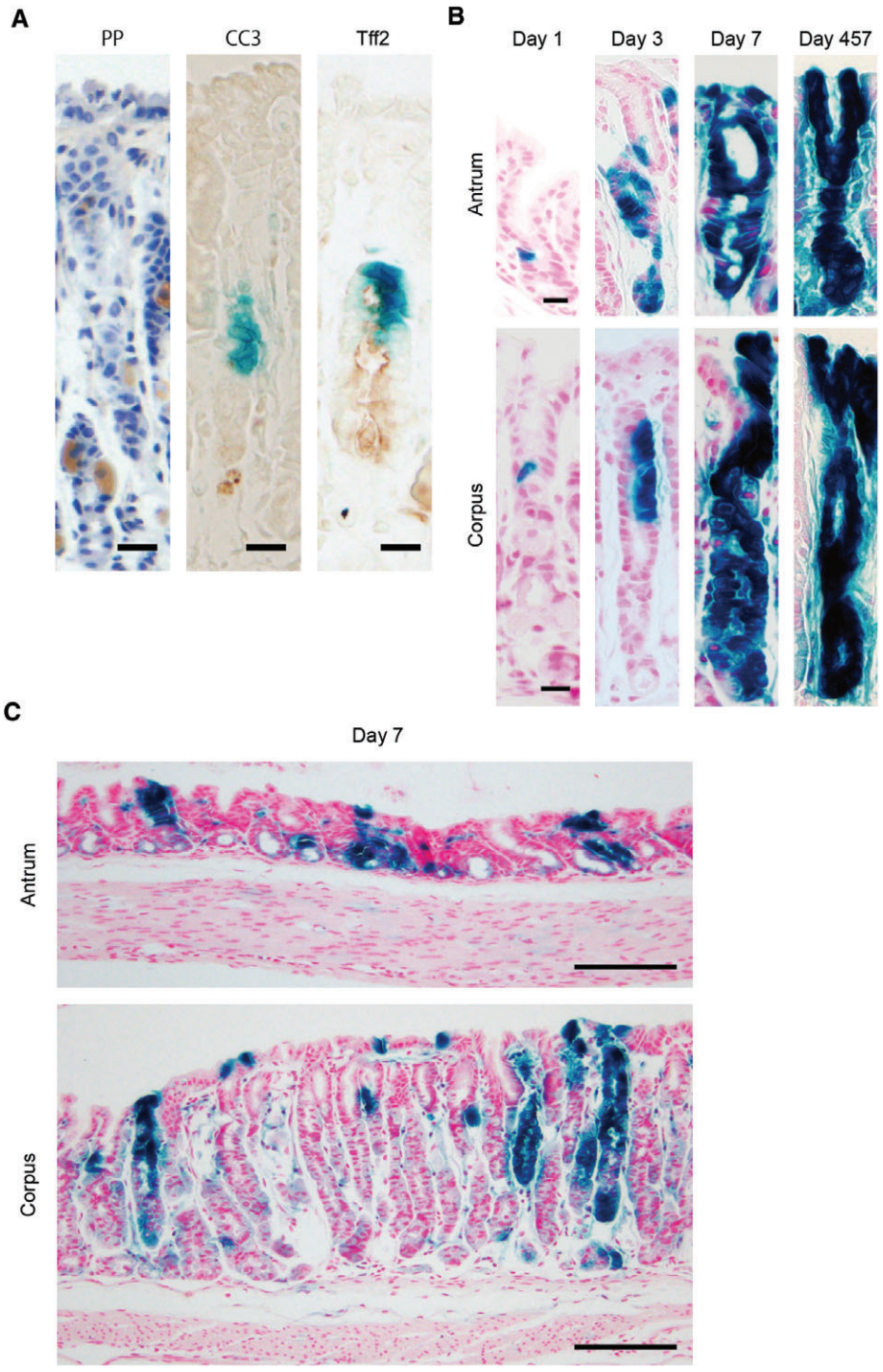

Figure 4. High-dose tamoxifen induced metaplasia and efficient production of progeny cells from Bmi1-expressing cells in the isthmus. (A) Injured gastric epithelium 3 days after 4 mg/mouse tamoxifen injection to 8-week-old mice. Immunohistochemistry for proton pump (PP), cleaved caspase 3 (CC3) and Tff2 within LacZ-stained gastric epithelium in *Bmi1-CreERT; Rosa26-LacZ* mice. Scale bars: 10 μm. (B) LacZ staining of the gastric epithelium in 8-week-old *Bmi1-CreERT; Rosa26-LacZ* mice 1, 3, 7 and 457 days after 4 mg/mouse tamoxifen injection. Scale bars: 10 μm. (C) LacZ staining of the gastric epithelium in 8-week-old *Bmi1-CreERT; Rosa26-LacZ* mice 1 week after 4 mg/mouse tamoxifen injection. Scale bars: 50 μm.

Rosa26-LacZ mice, according to reported protocols [2,18]. We administered 1 μM 4-hydroxytamoxifen (day 0) at 24 h after organoid preparation and after 22 h exchanged the gastric culture medium. Then we performed X-gal staining in the incubation dishes on days 2 and 6. We detected only one or two LacZ-positive cells in organoids established from the antrum and corpus on day 1 and observed these cells to be expanded on day 6 (Figure 6C). We next examined the requirement for Bmi1-expressing cells to maintain gastric organoids. We prepared gastric organoids from the gastric antrum and corpus epithelium of *Bmi1-CreERT; Rosa-iDTR* mice. At 22 h after 4-hydroxytamoxifen administration (day 0), we administered DT at 400 ng/500 μl for 36 h in

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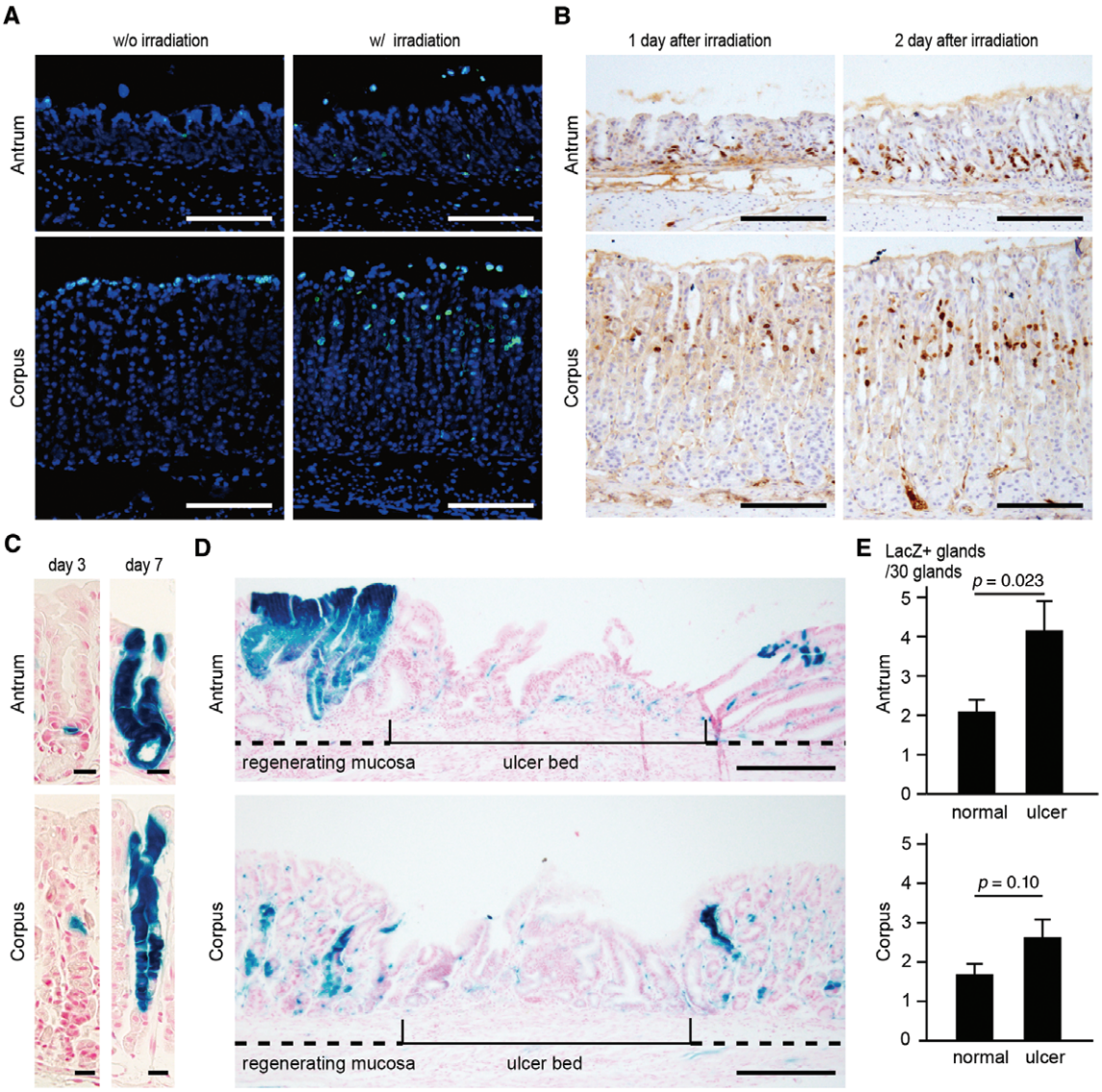

Figure 5. Bmi1 marked gastric stem cells during regeneration after irradiation- and acetic acid-induced mucosal injuries. (A) TUNEL staining in 8-week-old wild-type mice with or without 8 Gy irradiation, 1 day before sacrifice. Scale bars: 50 μm. (B) Immunohistochemistry for Ki67 1 or 2 days after 8 Gy irradiation to 8-week-old wild-type mice. Scale bars: 50 μm. (C) LacZ staining of 8-week-old *Bmi1-CreERT; Rosa26-LacZ* mice 3 and 7 days after 8 Gy irradiation. Tamoxifen was injected 1 day after irradiation. Scale bars: 10 μm. (D) LacZ staining of *Bmi1-CreERT; Rosa26-LacZ* mice 14 days after acetic acid-induced gastric ulcer injury. Tamoxifen was injected 2 days before the ulcer construction. Scale bars: 50 μm. (E) The number of LacZ-labeled traced glands per 30 glands from the edge of ulcer beds ($n = 5$).

the DT-treated group, or gastric culture medium in the non-DT-treated group, and counted grown organoids per 1 mm^2 field on day 5 (Figure 6D). The number of grown organoids was lower in the DT-treated group compared with the non-DT-treated group (Figure 6E). These data support the idea that Bmi1-expressing cells are stem/progenitor cells in the *ex vivo* gastric culture system.

Discussion

In this study we have shown the stem cell potential of Bmi1-expressing cells in mouse stomach. Bmi1-expressing cells in the isthmus have high proliferative activities and provide differentiated progeny bidirectionally towards the luminal and basal sides. Mucosal injuries induced by high-dose tamoxifen, irradiation or acetic acid induced rapid provision of progeny from Bmi1-expressing cells in the corpus. Gastric epithelium of both the antrum and the corpus was impaired by the ablation of Bmi1-expressing cells.

In the last decade, as lineage-tracing methods have been applied to digestive organs, considerable advances have been achieved in gastrointestinal stem cell research. For example, Lgr5 was reported as a marker for rapidly cycling stem cells located in the intestinal crypt base by such a method [19]. Subsequently, other stem cell markers, such as Bmi1, mTert, Ascl2, Olfm4, CD133, Hopx and Lrig1, were also reported to be intestinal stem cell markers [20–25]. Among them, Bmi1 was first reported to be expressed

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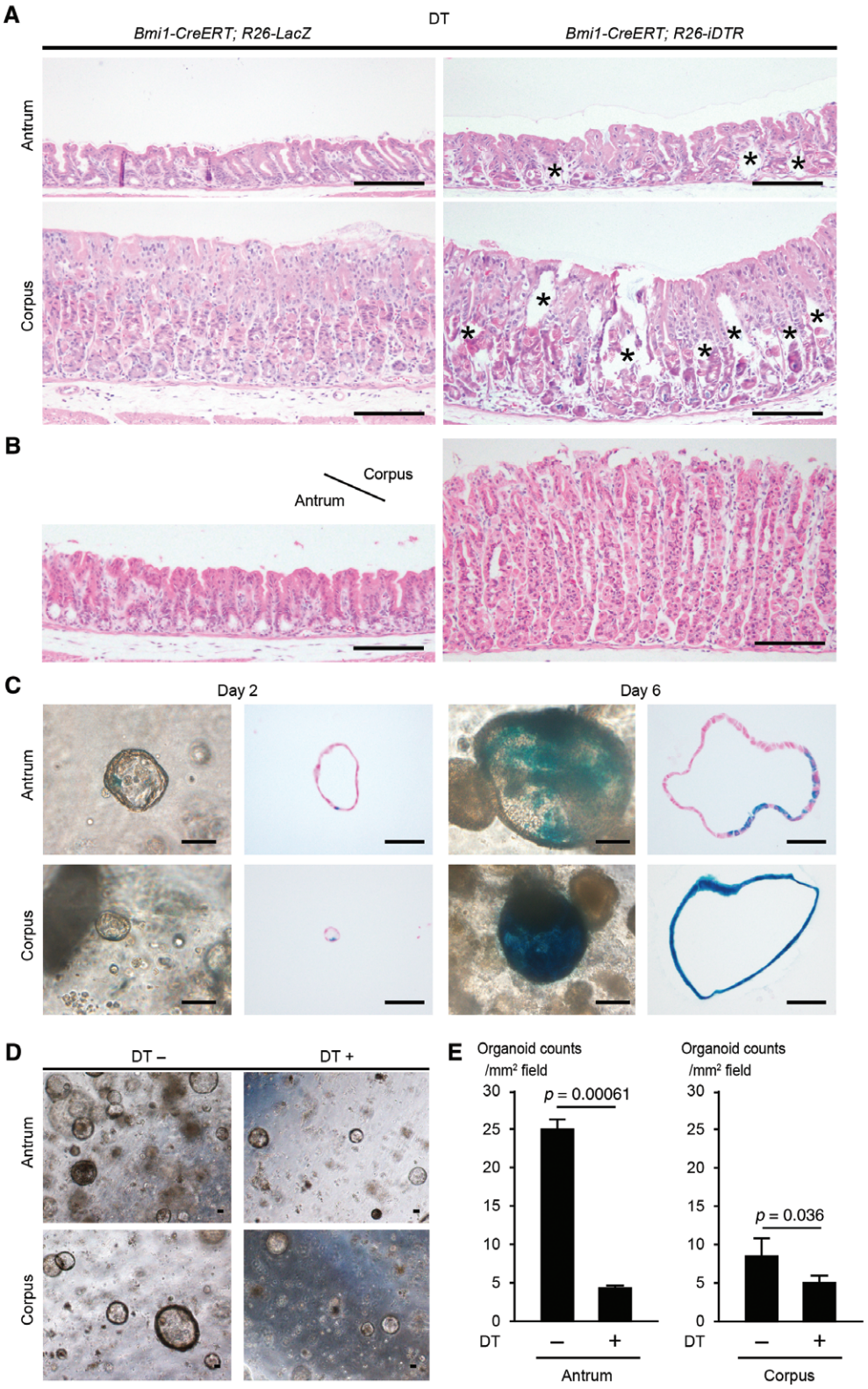

Figure 6. Ablation of Bmi1-expressing cells disturbed gastric epithelial homeostasis. (A) H&E staining of control *Bmi1-CreERT; Rosa26-LacZ* and *Bmi1-CreERT; Rosa26-iDTR* mice treated with tamoxifen and DT to specifically ablate Bmi1-expressing cells. Asterisks indicate injured epithelium. Scale bars: 50 μm. (B) H&E staining of *Bmi1-CreERT; Rosa26-iDTR* mice 1 week after treatment with tamoxifen and DT to specifically ablate Bmi1-expressing cells. Scale bars: 50 μm. (C) Lineage tracing of gastric organoids established from the gastric antrum and corpus epithelium using *Bmi1-CreERT; Rosa26-LacZ* mice. On day 0, 24 h after the organoid preparation, tamoxifen was administered for 22 h. On day 2, only one or two LacZ-positive cells were detected, whereas LacZ-positive cells expanded on day 6. (D) Ablation of Bmi1-expressing cells in gastric organoids from *Bmi1-CreERT; Rosa26-iDTR* mice. On day 0, 24 h after the organoid preparation, tamoxifen was administered for 22 h. The organoid was then incubated with or without DT for 36 h. Representative cultures on day 5 are shown. (E) The number of DT-treated live organoids per 1 mm^2 field derived from both the antrum and the corpus was significantly lower than that of non-DT-treated organoids on day 5 ($n = 3$). Scale bars: 50 μm.

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in the cells located in the +4 position of intestinal crypts, where quiescent label-retaining cells exist [20]. Previous reports demonstrated that Bmi1-positive quiescent stem cells in the intestine serve as reserve stem cells after Lgr5-positive stem cell injury [16] and that Lgr5-positive cells are required for regeneration after radiation injury [26]. On the other hand, another report has indicated that Bmi1 expression is scattered to intestinal cells, including Lgr5-positive stem cells, and that +4 label-retaining cells expressing Bmi1 are the secretory precursors [27]. Another study using $Bmi1^{GFP/+}$ mice also reported that Bmi1+ intestinal cells are probably enriched for enteroendocrine markers and are thought to be mainly homeostatic- and injury-inducible intestinal stem cells [28]. Thus, the stem cell potential of Bmi1-expressing cells is still under debate, even in the intestines, and their role in the stomach has not been reported.

In the stomach, Lgr5 was the first reported stem cell marker. Lgr5-expressing cells were located in the base of antral mucosa and showed 29% co-staining with Ki67 [3]. Another antral stem cell marker, Cckbr, was expressed in the +4 position, which is the lower part of the isthmus, and showed 30% co-staining with Ki67 [6]. In our study, Bmi1-expressing cells were located in the +5 to +8 position in the gastric antrum, and were co-stained with Ki67 at 71%. These data suggest that most Bmi1-expressing cells were different from Lgr5- and Cckbr-positive cells in terms of cell position and proliferative potential.

In the isthmus of the corpus, which had been regarded as a gastric stem cell zone, Mist1 was the first stem cell marker identified by lineage-tracing experiments with 3 mg tamoxifen administration [7]. Because Mist1-positive stem cells rarely expressed proliferative markers (1.1% co-staining ratio with Ki67) [7], it is reasonable that Bmi1-expressing cells are different cell populations from Mist1-positive cells. On the other hand, another gastric stem cell marker, eR1-positive isthmus cells, had a high ratio of co-staining with Ki67 (80%) in the corpus [8]. Although eR1-positive cells and Bmi1-expressing cells share high Ki67-positive ratios, our mouse studies showed that they were different cell populations.

There is a pitfall to investigating gastric stem cell potential by lineage-tracing experiments, because gastric epithelium is sensitive to high-dose tamoxifen [10] in terms of parietal cell damage and metaplastic change. Troy- and Lgr5-positive cells were both reported as reserve stem cells of gastric corpus, located in the base [5,9]. *Lgr5-2A-CreERT2* mice had a highly efficient induction rate, but low-dose tamoxifen administration (0.1 mg/g body weight) showed rare lineage-tracing events in gastric corpus [9]. Administration of high-dose tamoxifen (0.27 mg/g body weight) enabled highly efficient lineage tracing over several months, but resulted in parietal cell loss and mucosal injury [9]. Consistently, the efficiency of Bmi1 lineage induction was dependent on the dose of tamoxifen. In this study, although Bmi1-expressing cells provided progeny in the whole gastric gland for several months after 0.1 mg/g body weight low-dose tamoxifen, they did provide progeny more rapidly and contribute to the metaplasia-like regeneration in 7 days after 4 mg/mouse high-dose tamoxifen injection, irrespective of parietal cell damage. Bmi1-expressing cells did not overlap with chief cells, which contain Troy- and Lgr5-positive cell populations, and thus contributed to metaplastic changes more rapidly than Troy- and Lgr5-positive cells [5,9]. Although gastric epithelium may present difficulty in the interpretation of stemness, it is worth noting that Bmi1-expressing cells have different characteristics from cells expressing Lgr5, Cckbr, Troy, Mist1 or eR1 in terms of localization, cell cycling and sensitivity for tamoxifen. At present, we could not examine the colocalization of Bmi1 and other reported stem cell markers other than Lgr5 and eR1. Further studies may clarify the hierarchy or overlaps between gastric stem cell markers in future.

In conclusion, Bmi1 is a stem cell marker expressed in the isthmus of gastric antrum and corpus in mice. Bmi1-expressing cells provided progeny bidirectionally towards the luminal and basal sides, which concurs with the historical concept of gastric stem cells [29–32]. Also, Bmi1-expressing cells played an important role in regeneration after gastric epithelium injury and were indispensable for the homeostasis of gastric epithelium.

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Author contributions statement

TY, OA, SO, YH, YM, YY, YN and HS conceived and designed the study. KK and YS provided essential materials. TY, OA, SO and YH performed the experiments and analyzed the data. TY wrote the manuscript and AF, TC and HS revised it.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Supplementary figure legends

Figure S1. Bmi1-expressing cells were mainly located in the isthmus of stomach and were unique from other differentiated cells after high-dose tamoxifen-induced gastric mucosal injury

Figure S2. Bmi1-expressing cells exhibit stem cell properties in gastric injury models

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