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Establishment of a long-term three-dimensional primary culture of mouse glandular stomach epithelial cells within the stem cell niche

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ABSTRACT

Compared to the small intestine and colon, little is known about stem cells in the stomach because of a lack of specific stem cell markers and an in vitro system that allows long-term culture. Here we describe a long-term three-dimensional (3D) primary gastric culture system within the stem cell niche. Glandular stomach cells from neonatal mice cultured in collagen gel yielded expanding sphere-like structures for 3 months. The wall of the gastrospheres consisted of a highly polarized epithelial monolayer with an outer lining of myofibroblasts. The epithelial cells showed a tall columnar cell shape, basal round nuclei, and mucus-filled cytoplasm as well as expression of MUC5AC, indicating differentiation into gastric surface mucous cells. These cells demonstrated the features of fully differentiated gastric surface mucous cells such as microvilli, junctional complexes, and glycogen and secretory granules. Fewer than 1% of cultured epithelial cells differentiated into enteroendocrine cells. Active proliferation of the epithelial cells and many apoptotic cells in the inner lumen revealed the rapid cell turnover in gastrospheres in vitro. This method enables us to investigate the role of signaling between cell–cell and epithelial–mesenchymal interactions in an environment that is extremely similar to the in vivo environment.

1. Introduction

The gastrointestinal mucosa is exposed to a harsh external environment, and homeostasis is maintained by a variety of mechanisms. Epithelial cells of the gastric mucosa include surface mucous (foveolar) cells, mucous neck cells, parietal cells, zymogenic (chief) cells, and enteroendocrine cells. These different cell types are organized into repeating flask-like structures called glands that are surrounded by supporting stromal cells [1,2]. The vertical unit of the gastric mucosa is composed of the pit, isthmus, neck, and base [1,2].

With advances in stem cell research, molecular markers of stem cells have been reported for the small intestine and colon [3,4]. However, little is known about gastric stem cells because neither specific stem cell markers nor an in vitro system that allows long-term culture have been established. Therefore, current studies of gastric stem cells rely on the use of cell lines or in vivo studies in genetically manipulated mice, which are expensive and time consuming [5].

Although several researchers have generated cultures of primary gastric epithelium [6–11], these cells can be maintained for only about a week at most. Barker et al. [5] reported a method of primary gastric long-term culture from single Leucine-Rich G Protein-coupled receptor 5 (Lgr5)$^+$ cells. Their method is an invaluable tool, but it requires many additional reagents to maintain and differentiate single, sorted Lgr5$^+$ cells into pyloric gland organoids in vitro. Furthermore, technical proficiency is required for sorting a single Lgr5$^+$ cell from the *Lgr5-EGFP-ires-CreERT2* knock-in mouse, suggesting that their method may be not physiologically relevant.

To solve these problems, we have established a three-dimensional (3D) primary gastric culture system applying our previous method for primary intestinal stem cell niche culture [12]. In this system, we demonstrate the maintenance of gastric stem or progenitor cells within the stem cell niche over the long term without addition of special reagents. Because our system recapitulates

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the in vivo microenvironment more easily and rapidly, this system will be useful not only in gastric stem cell research but also for investigating various diseases of the gastric mucosa.

2. Materials and methods

2.1. 3D primary gastric culture system

We applied the 3D culture system previously reported by Ootani et al. [12]. This system maintains cultured cells that are embedded in a collagen gel under an air–liquid interface environment (Fig. 1A). Cellmatrix type I-A (Nitta Gelatin Inc., Osaka, Japan), Ham's F-12, and sterile reconstitution buffer (2.2 g $NaHCO_3$ in 100 ml of 0.05 N NaOH and 200 mM HEPES) were mixed at a ratio 8:1:1. This reconstituted collagen solution (1.2 ml) was poured into an inner 30-mm diameter dish (Millicell culture plate inserts, PICM03050, Millicell-CM, Millipore, Billerica, MA, USA). After 30 min at 37 °C, the collagen gel in the inner dish had solidified, and the tissue was prepared. The glandular stomach was removed on ice from postnatal day 2 C57BL/6J mice and immediately immersed and washed in phosphate-buffered saline (PBS). The washed tissue was minced within 5 min and mixed in reconstituted collagen solution as described above. The final minced tissue was under 0.3 mm^3. The tissue from one neonatal mouse per dish was mixed. All work from tissue removal to this step was done on ice. The cell-containing collagen gel (1.2 ml) was poured onto the collagen gel in the inner dish, which was placed in a 60-mm diameter outer dish. After the cell-containing gel solidified, 2.0 ml Ham's F12 supplemented with 20% fetal bovine serum and 50 μg ml^{-1} gentamicin was poured into the outer dish, which was covered and incubated at 37 °C in a humidified atmosphere of 5% CO_2 in air. Culture medium was replaced every 7 days. The experimental design was approved by the Animal Care Committee of the Nagoya City University Animal Research Institute, and the animals were cared for in accordance with institutional guidelines, in compliance with the instructions of the Health, Labour and Welfare Ministry concerning animal experiments.

2.2. Histology and histochemistry

Cultured cells in collagen gel were fixed in 10% formalin, processed routinely, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). To detect mucous substances, sections were stained with periodic acid-Schiff (PAS).

2.3. Immunohistochemistry

Immunohistochemical staining was carried out with antibodies against the following antigens: MUC5AC, MUC6, CD10 (1:100, Novocastra Laboratories, Newcastle upon Tyne, UK), MUC2 (1:100, Santa Cruz Biotechnology, CA, USA), chromogranin A

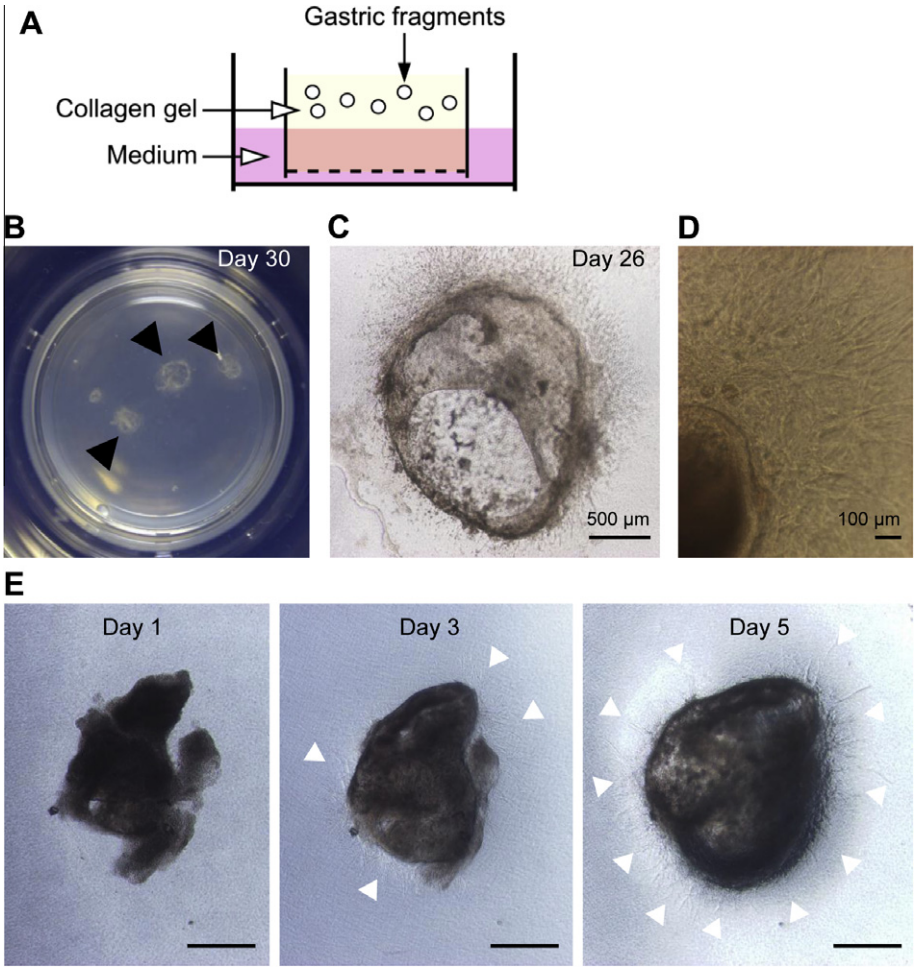

Fig. 1. Long-term 3D culture of glandular stomach. (A) Schematic representation of the 3D culture assembly. The gastric cultures are maintained in collagen gels under an air–liquid interface microenvironment. (B) Black arrowheads indicate gastrospheres at 30 days in a 3-cm inner dish. (C) The gastrospheres grow with sufficient outer growth of myofibroblasts at day 26. (D) Spindle-shaped myofibroblasts surround the outer wall of the gastrosphere. (E) The growth of myofibroblasts (white arrowheads) is essential for the growth of the gastrospheres. The same field is shown. Scale bars, 500 μm.

(1:1000, Yanaihara Institute Inc., Fujinomiya, Japan), anti-Proton Pump/H^+, K^+-ATPase (MBL Co., Ltd., Nagoya, Japan), proliferating cell nuclear antigen (PCNA) (1:100, Dako, Glostrup, Denmark), α-smooth muscle actin (SMA) (1:200, Epitomics, Burlingame, CA, USA), cytokeratin (Nichirei, Tokyo, Japan), and single-stranded (ss) DNA (1:400, IBL Co., Ltd., Fujioka, Japan). The precise procedures for immunohistochemical techniques were performed as described [13,14]. As a positive control, normal mouse stomach and intestine were used for immunohistochemistry. The controls always gave positive results in a cell type-specific manner.

2.4. Transmission electron microscopy

For transmission electron microscopy, samples were fixed with 2.5% glutaraldehyde and 1% osmic acid, dehydrated with alcohol, and embedded in epoxy resin as described previously [11].

2.5. Cell proliferation

To calculate the percent of $PCNA^+$ cells, 1000 cultured epithelial cells were counted, and the percent of $PCNA^+$ nuclei were calculated. The data obtained from four to five independent experiments were analyzed with analysis of variance. Results were expressed as means ± SEM.

3. Results

3.1. Cultured glandular stomach cells from neonatal mice

3D culture of glandular stomach from neonatal mice within a collagen gel with an air–liquid interface (Fig. 1A) yielded expanding cystic structures (termed gastrospheres, Fig. 1B) with outer spindle cell growth (Fig. 1C and D). All gastrospheres with sufficient outer spindle cells (more than half of the culture attempts) showed growth for more than 20 (20–80) days in vitro (Fig. 1E).

3.2. Histology and immunohistochemistry

The wall of the gastrospheres consisted of a polarized epithelial monolayer with an apical, inner luminal surface and a basal outer surface in close proximity to spindle-shaped cells and the collagen matrix (Fig. 2A–C). The epithelial monolayer was positive for cytokeratin (Fig. 2D), and the outer lining spindle cells in the matrix were positive for the myofibroblast marker α-SMA (Fig. 2E). A portion of the wall showed a pit-like structure, suggesting morphological differentiation of the gastric mucosa (Fig. 2B). The cultured gastric epithelial cells showed a tall columnar shape, basally situated round nuclei, and clear cytoplasm that was filled with mucus (Fig. 3A). The intracytoplasmic mucus stained strongly with PAS (Fig. 3B). These cells were positive for MUC5AC, indicating differentiation into gastric surface mucous cells (Fig. 3C). Fewer than 1% of the epithelial cells were positive for the enteroendocrine cell marker chromogranin A (Fig. 3D). The epithelial cells showed no expression of the parietal cell marker H^+/K^+-ATPase, the mucous neck cell marker MUC6, the absorptive cell marker CD10, or the goblet cell marker MUC2 (data not shown). PCNA staining demonstrated active proliferation of the cultured gastric epithelial cells (Fig. 3F), and 30.4 ± 7.2% of epithelial cells were PCNA positive. Some of the $PCNA^+$ cells showed a similar morphology as the proliferating zone of the isthmus in the gastric gland in vivo (Fig. 3E and F). Immunohistochemistry for ssDNA revealed many apoptotic cells in the inner lumen (Fig. 3G). These results revealed the rapid turnover of gastric epithelial cells in culture.

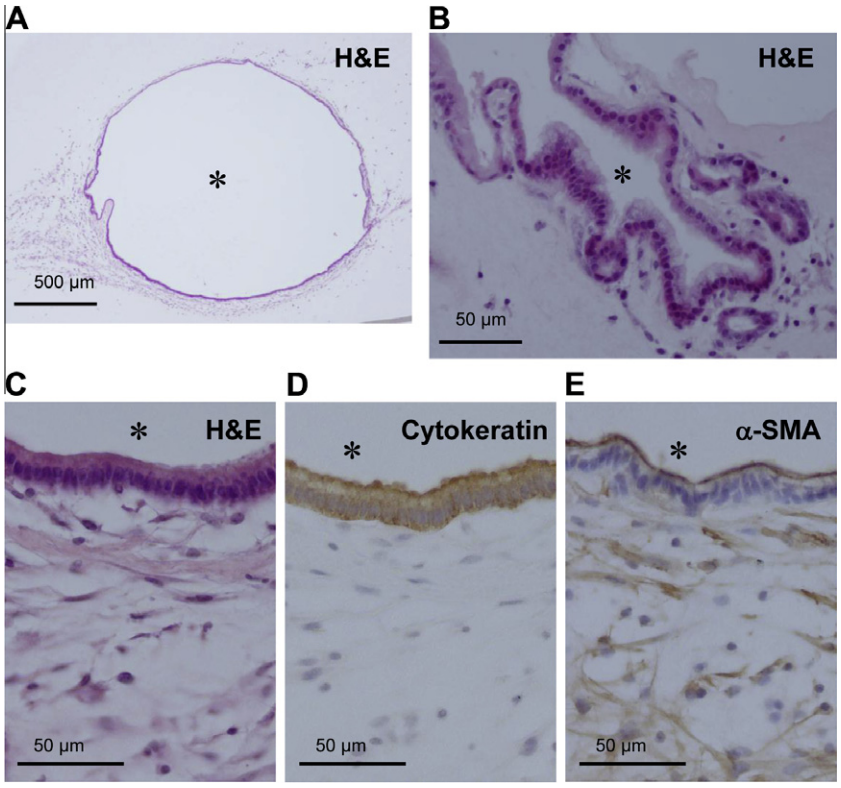

Fig. 2. A gastrosphere at culture day 20. (A) Low-power view of the gastrosphere. (B) Part of the wall shows a pit-like structure that is morphologically highly differentiated. (C) The wall of the gastrosphere consists of a monolayer of polarized, tall columnar epithelial cells with an outer lining of spindle-shaped cells. (D) The epithelial monolayer of the gastrosphere wall is positive for cytokeratin. (E) The outer lining of spindle-shaped cells in the collagen matrix is positive for the myofibroblast marker α-smooth muscle actin. (C), (D), and (E) are the same field. Asterisk indicates the lumen of the sphere.

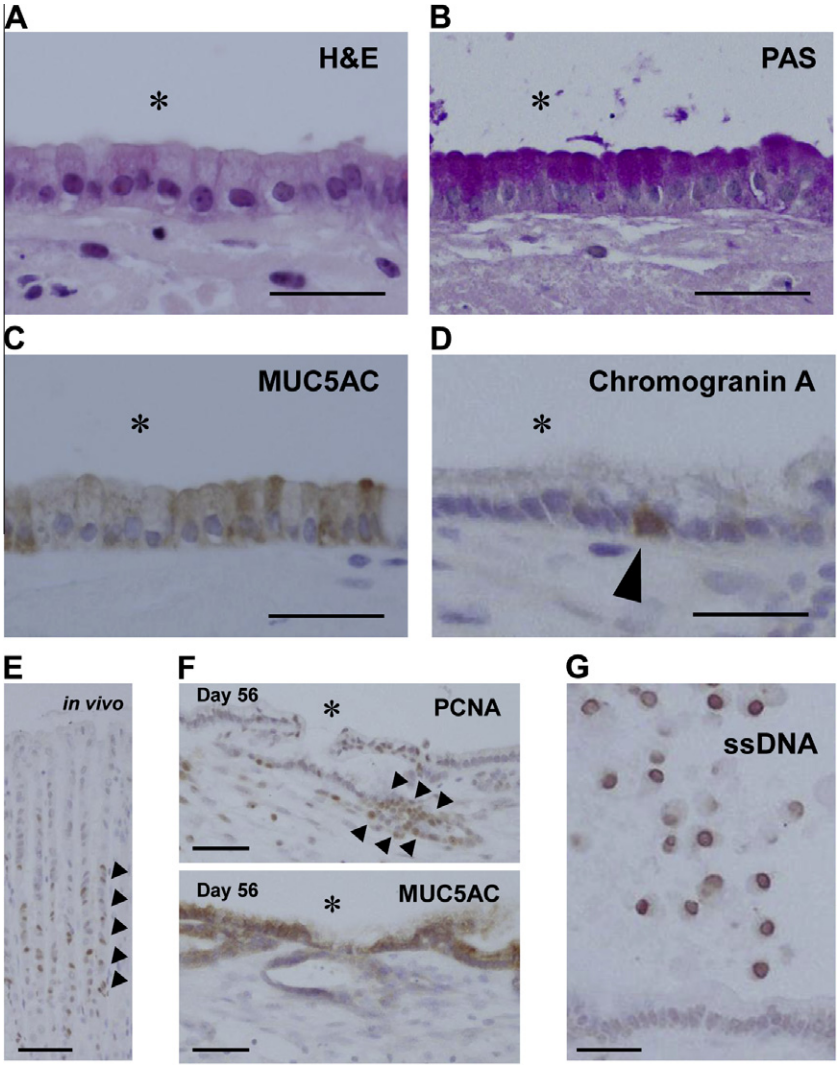

Fig. 3. Differentiation and proliferation of gastrospheres. (A), (B), and (C) are the same field at culture day 26. (A) The cultured gastric epithelial cells show a tall columnar shape, basally situated round nuclei, and clear cytoplasm. (B) The intracytoplasmic mucus is stained strongly with PAS. (C) The gastric surface mucous cell marker MUC5AC is expressed in the differentiated epithelial cells. (D) At culture day 56, cells positive for chromogranin A are observed in the gastric epithelium (arrowhead). (E) Corpus region of adult mouse. PCNA staining revealed proliferating zone (arrowheads) in vivo. (F) At culture day 56, PCNA staining shows a proliferating zone (arrowheads) in the gastrospheres, which is similar to the isthmus in vivo. The foveolar differentiated epithelial cells demonstrate expression of MUC5AC but not PCNA, mimicking the gastric surface mucosa in vivo. (G) Immunohistochemistry for ssDNA reveals many apoptotic cells in the inner lumen of the gastrospheres. Asterisk indicates the lumen of the sphere. Scale bars, 50 μm.

3.3. Transmission electron microscopy

Ultrastructural examination revealed fully differentiated microstructures in cultured gastric epithelial cells. The wall of the gastrospheres consisted of a differentiated epithelial monolayer with an outer lining of myofibroblasts (Fig. 4A). Highly polarized, tall columnar epithelial cells showed stubby microvilli protruding from the apical membrane (Fig. 4B). They contained many secretory granules in the apical cytoplasm and many glycogen granules in the supra-nuclear region (Fig. 4B and C). The lateral membranes of each cell were attached to those of their neighbors with junctional complexes at their apical regions (Fig. 4D).

4. Discussion

We have established a long-term 3D primary culture of gastric mucosa applying our previous method for primary intestinal stem cell niche culture [12]. In the past, several researchers have reported methods for primary gastric culture. In 1982, Terano et al. reported a method for culturing rat gastric fundic mucosa in a monolayer [6], but this only allowed cells to be cultured for 2 weeks. Therefore, the process of cell differentiation and steric proliferation could not be observed. In other reports of primary monolayer culture methods using human and animal specimens, cells could only be cultured for a few days to 2 weeks at most, and repeated passaging of these cultures was not possible [7–10]. In 2003, we established a 3D reconstructed primary culture system of gastric surface mucosa that enables maintenance of fully differentiated gastric surface mucous cells [11]. We found that cultured gastric epithelial cells require interaction with stromal myofibroblasts and an air–liquid interface microenvironment to differentiate into surface mucous cells in vitro [11,15]. These methods, however, maintain cellular viability for less than 14 d. An appropriate in vitro culture system of primary gastric epithelium that sustains stem cell function has not been established. Therefore,

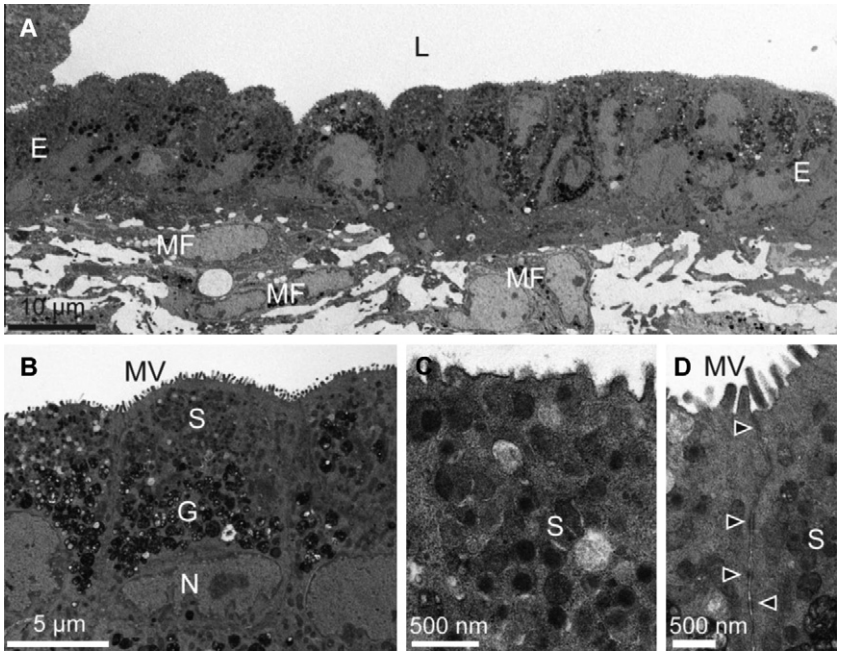

Fig. 4. Electron micrographs of the cultured gastric mucosa (day 30). (A) The wall of the gastrospheres consists of an epithelial monolayer (E) and an outer lining of myofibroblasts (MF). (B, C) Highly polarized, tall columnar epithelial cells show microvilli (MV) at the apical surface, and secretory granules (S) and glycogen granules (G) in the apical cytoplasm. (D) Well-organized junctional complexes (arrowheads) are seen in the basolateral domain anchoring adjacent epithelial cells. L, lumen of the gastrosphere; MF, myofibroblast; MV, microvilli; S, secretory granules; G, glycogen granules; N, nucleus.

current research in gastric stem cells, epithelial homeostasis, and gastric cancer relies heavily on the use of animal cell lines or in vivo genetically manipulated mouse models [5]. These research materials are expensive and time-consuming to generate. With our culture system, in more than half of our culture attempts, we were able to observe gastric epithelial spheres growing for $\geqslant$30 days, following outer myofibroblast growth. Without special additional reagents in the outer dish, the cultured gastrospheres showed differentiation into MUC5AC$^+$ surface mucous cells with pit-like formations. Our method demonstrated better reproducibility using neonatal murine stomach without additional genetic manipulation and made culture of primary gastric cells for $\geqslant$30 days fast and easy.

Tissue stem cells are defined by the two specialized properties of self-renewal and multi-lineage differentiation. These two properties contribute to tissue homeostasis and injury repair. Adult or somatic stem cells generally have limited functions outside their niche [16]. Interaction between stem cells and the stem cell niche is the basis of tissue homeostasis [17–19]. In our system, culture within the stem cell niche is possible by embedding the entire minced glandular stomach of neonatal mice in the collagen gel. The presence of many apoptotic cells positive for ssDNA in the sphere lumen and the rate of PCNA$^+$ cells revealed rapid turnover and proliferation of gastric epithelial cells in a long-term culture, strongly suggesting that gastric stem cells are contained in the cultured epithelial cells. The gastric stem cells may automatically select the required stem cell niche in vitro, making long-term culture possible.

Epithelial cells of the gastric mucosa are organized in vertical flask-shaped structures consisting of a pit region, isthmus, neck, and gland base [1,2]. In the corpus of the stomach, the glands are long and composed of several epithelial cell types, including surface mucous cells or pit cells, mucous neck cells, acid-secreting parietal cells, zymogenic or chief cells, and enteroendocrine cells [1,2]. In the antrum, glands are composed mainly of mucus-secreting cells as well as enteroendocrine cells [1,2]. The turnover time of each type of gastric epithelial cell is different. The oxyntic pit-gland unit includes surface mucous cells, zymogenic cells, and parietal cells, which migrate outwards, inwards, and in both directions, respectively; their turnover times average 3, 194, and 54 days, respectively [20]. The mucous units of the pyloric antrum are populated by surface mucous cells that migrate outwards and pyloric gland cells that migrate inwards; their turnover times are about 3 and 1–60 days, respectively [20]. With histology and immunohistochemistry, we confirmed the process of MUC5AC$^+$ surface mucous cells forming pit-like structures, followed by formation of a monolayer of columnar epithelium. However, the cultured epithelial cells were negative for MUC6, a marker of mucous neck cells or pyloric gland cells. These results may represent the different turnover times of mucus-producing cells in the pit and gland of the gastric mucosa. Similarly, the epithelial cells did not express H^+/K^+-ATPase in the gastrospheres. Considering the turnover time, further long-term culture may be required to introduce glandular differentiation in vitro.

In the intestine, emerging evidence indicates that slowly cycling (quiescent) stem cells dynamically interconvert with more rapidly cycling (active) stem cells [21–24]. Although several investigators have reported candidate gastric stem cell markers [5,25–31], only cells positive for two of these markers, villin and Lgr5, have been shown to be capable of replenishing entire gastric pyloric glands [5,30]. The cell cycles of villin$^+$ stem cells and Lgr5$^+$ stem cells seem to be different, but the relationship between these two populations has not yet been reported. Further, a marker for stem cells of the gastric corpus gland has not been identified. The major feature of our 3D culture system is the ability to reconstitute normal tissue to induce epithelial cells with appropriate polarity that interact with stromal myofibroblasts. This method enables us to investigate not only the effect of signaling between cell–cell and epithelial–mesenchymal interactions but the relationship between villin$^+$ and Lgr5$^+$ stem cells in an environment that is extremely similar to the in vivo environment.

In the stomach, *Helicobacter pylori* infection is well known to induce a shift from the gastric to the intestinal phenotype in both cancerous and non-neoplastic mucosa [32]. Gastric and intestinal phenotypic expression is important for the histogenesis of cancers of the digestive system such as stomach, colorectal, and pancreatic cancers [13,33]. Expression of caudal-related homeobox gene 2 (Cdx2), an intestinal-specific gene, is important for the intestinalization in both cancerous and non-neoplastic mucosa of the stomach, and *H. pylori* infection triggers ectopic Cdx2 expression in the stomach [34]. Cdx2 expression is also correlated with prognosis of stomach and pancreatic cancers, and Cdx2-positive groups have better outcomes than patients that are negative for Cdx2 [34]. However, the mechanism of transformation from the gastric to the intestinal phenotype remains unclear, because an appropriate long-term culture system to maintain the gastric phenotype has not been established. Using our culture system, we will confirm the dynamic change from the gastric to the intestinal phenotype by overexpression or knockdown of individual essential genes, including Cdx2, or with *H. pylori* administration in vivo. Further studies to elucidate the mechanism of the shift from the gastric to the intestinal phenotype are currently being performed in our laboratories. We hope these experiments will lead to further understanding of carcinogenesis and elucidating new approaches to anti-cancer therapy such as intestinal differentiation induction therapy in the digestive system, especially in the stomach.

In conclusion, using our 3D culture system, a long-term primary stomach cell culture with interaction of the stem cell niche was established. This system is capable of recapitulating various environments by adding various reagents to the outer dish, and we can thus dynamically observe various interactions. This culture method should be useful for understanding stem cells, understanding the interaction between stem cells and their niche, revealing gastric stem cell markers, and elucidating the cause of gastric diseases.

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