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CD133 is a marker of gland-forming cells in gastric tumors and Sox17 is involved in its regulation

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CD133 is a universal marker of tissue stem/progenitor cells as well as cancer stem cells, but its physiological significance remains to be elucidated. Here we examined the relationship between expression of CD133 and features of gastric epithelial cells, and found that CD133-positive (CD133[+]) tumor cell lines formed well-differentiated tumors while CD133-negative (CD133[–]) lines formed poorly differentiated ones when subcutaneously injected into nude mice. We also found that CD133(+) and CD133(–) cell populations co-existed in some cell lines. FACS analysis showed that CD133(+) cells were mother cells because CD133(+) cells formed both CD133(+) and CD133(–) cells, but CD133(–) cells did not form CD133(+) cells. In these cell lines, CD133(+) cells formed well-differentiated tumors while CD133(–) cells formed poorly differentiated ones. In human gastric cancers, CD133 was exclusively expressed on the luminal surface membrane of gland-forming cells, and it was never found on poorly differentiated diffuse-type cells. Considering that poorly differentiated tumors often develop from well-differentiated tumors during tumor progression, these results suggest that loss of expression of CD133 might be related to gastric tumor progression. Microarray analysis showed that CD133(+) cells specifically expressed *Sox17*, a tumor suppressor in gastric carcinogenesis. Forced expression of *SOX17* induced expression of *CD133* in CD133(–) cells, and reduction of *SOX17* caused by siRNA in CD133(+) cells induced a reduction in the level of *CD133*. These results indicate that Sox17 might be a key transcription factor controlling CD133 expression, and that it might also play a role in the control of gastric tumor progression. (*Cancer Sci* 2011; 102: 1313–1321)

Gastric adenocarcinomas are the second leading cause of cancer-related mortality in the world.[1] It is well established that environmental factors including *Helicobacter pylori* infection[2] and excessive intake of salt[3] are associated with tumorigenesis, but the molecular mechanism regulating gastric epithelial growth and differentiation has not been fully elucidated. Pathologically, gastric cancer can be divided into well-differentiated (intestinal) and poorly differentiated (diffuse) types according to the presence or absence of tubular structures.[4] The former is considered to occur through a stepwise change from chronic gastritis to atrophic gastritis, followed by spasmolytic polypeptide-expressing metaplasia and intestinal metaplasia, resulting in dysplasia and carcinomas,[5] but the latter through a less well-characterized sequence of events including a dysfunction of E-cadherin.[6]

Gastric cancers show great histological diversity, which tends to increase with deeper invasion and increased tumor diameter.[7] Thus, it is usual that they include a mixture of the well-differentiated and poorly differentiated types. In such cases, they are classified based on the predominant histological type according to the Japanese Classification of Gastric Carcinoma.[8] It has been a big problem how such mixed-type cancers are formed, and how it is related to the progression of gastric cancers. By detailed analysis of early and advanced gastric cancers, Ikeda *et al.*[9] suggested that the predominant histological type changed from well-differentiated to poorly differentiated types with the progression of tumors. On analysis of early gastric cancers, Saito *et al.*[10] indicated that small well-differentiated adenocarcinomas transformed into poorly differentiated tumors during their progression. Consistent with these, Takizawa and Koike[11] presented evidence showing that poorly differentiated tumor cells (signet ring cells) were formed from well-differentiated gastric carcinomas. Thus, it is probable that these two tumor types are related, and that some poorly differentiated tumor cells are derived from well-differentiated tumors through an unknown mechanism. It remains to be determined by what mechanism poorly differentiated tumor cells are induced from well-differentiated tumors. It has been difficult to analyze because no experimental systems have been reported in which the characteristics of gastric epithelial cells change from well-differentiated to poorly differentiated types *in vitro*.

We previously reported that Runx3, a runt domain transcription factor, is a major growth regulator of gastric epithelial cells, and that a lack of RUNX3 function is causally related to the genesis and progression of human gastric cancer.[12] We have so far established several *Runx3−/−p53−/−* gastric epithelial cell lines, all of which are tumorigenic, and some of which exhibit intestinal-type differentiation *in vivo*[13] and *in vitro*.[14] It remains to be solved how their differentiation is regulated, and whether changes in their differentiation are related to the progression of tumors.

CD133 is a transmembrane glycoprotein first identified on human hematopoietic stem cells by using a monoclonal antibody, AC133. Its murine counterpart, Prominin-1, has also been found on embryonic neuroepithelia and various other epithelia in embryos.[15] Recently, CD133/Prominin-1 (CD133) has been reported to be a marker of cancer stem cells in brain,[16,17] lung,[18] pancreas,[19] prostate,[20] colon[21,22] and liver[23] tumors. However, controversial results have been reported regarding the significance of CD133 in gastric tumorigenesis since Smith *et al.*[24] reported that 47–55% of gastric tumor samples were positive for CD133, while Boeg and Prinz[25] found that expression of CD133 decreased with the progression of carcinogenesis in gastric cancers.

Here, we examined the relationship between expression of CD133 and the differentiation potency of *Runx3−/−p53−/−* gastric epithelial cells, and obtained evidence that loss of CD133 expression might be related to the phenotypic change of tumors from well-differentiated to poorly differentiated ones. Our analysis also indicated that Sox17, a key regulator of the

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development of a definitive endoderm[26,27] might be involved in the control of CD133 expression in gastric epithelial cells.

Materials and Methods

Cell culture and FACS analysis. The establishment of GIF-3, -5, -11 and -14 *Runx3−/−p53−/−* mouse gastric epithelial cell lines was described in a previous paper,[13] and the cells were cultured in DMEM + 10% bovine serum (Handai-Biken, Osaka, Japan). KATO-III, MKN45 and MKN74 human gastric cancer cells were cultured in RPMI1640 + 10% FBS. Single cell suspensions were stained with phycoerythrin (PE)-labeled anti-CD133 antibody (eBioscience, San Diego, CA, USA; clone13A4, 1:100) at 4°C for 30 min, and were sorted with a FACSVantage (Becton-Dickinson, Franklin Lakes, NJ, USA).

Characteristics of CD133(+) and CD133(–) cells *in vitro* and *in vivo*. The characteristics of CD133-positive (CD133[+]) and CD133-negative (CD133[−]) cells were compared *in vitro* by culturing them at the air-liquid interface after mixing them with Matrigel (BD) and clotting the mixtures on filters (HAWP; Millipore, Billerica, MA, USA), as described previously.[28] Some cells with Matrigel were subcutaneously injected into nude mice (SRL Japan, Shizuoka, Japan) to examine their differentiation *in vivo*. Tissues were fixed with 95% ethyl alcohol, embedded in paraffin, sectioned at 5 μm and stained with hematoxylin–eosin (HE) or periodic acid-Schiff (PAS)-hematoxylin.

Immunohistochemical analyses. Paraffin-embedded human gastric tumor tissues were sectioned at 5 μm, and used for immunohistochemical staining of CD133 according to the method of Kojima *et al.*[29] Briefly, sections were deparaffinized, and endogenous peroxidase activity was inhibited by 0.3% hydrogen peroxidase solution in methanol. For antigen retrieval, the slides were treated with 1 mM ethylenediaminetetraacetic acid buffer (pH 8.0) at 95°C for 20 min in a microwave oven and allowed to cool for 1 h at room temperature. Each slide was then treated overnight at 4°C with anti-CD133 antibody (Miltenyi Biotec, Auburn, CA, USA; clone AC133, 1:100), followed by incubation with Histofine Simple Stain Max PO (Nichirei, Tokyo, Japan). The localization of antigen was revealed using 3, 3′-diaminobenzidine tetrahydrochloride as a chromogen, and the slide was counterstained with neutral red.

Some were also stained with anti-SOX17 rabbit polyclonal antibodies (Millipore Cat# 09-038) after antigen retrieval with 10 mM citrate buffer (pH 6.0) for 1 min at 120°C, according to Nonaka.[30] Other staining methods were similar to those for CD133 as described above. The relationship between CD133- and SOX17-expressing cells was examined by staining adjacent sections.

Microarray analysis of gene expression of CD133(+) and CD133(–) GIF-5 cells. RNA was extracted from CD133(+) and CD133(−) GIF-5 cells, and total RNAs were labeled with a Low RNA Input Linear Amp kit (Agilent Technologies, Santa Clara, CA, USA). Differentially labeled cRNAs from CD133(+) and CD133(−) cells were compared using an oligoDNA microarray for the whole mouse genome in a dye swap experiment (Agilent Technologies), according to the instructions of the manufacturer. Reproducible results consistently obtained on two independent analyses were used for further analysis. Detailed information concerning experimental procedures as well as all microarray data discussed in this publication have been deposited in the Gene National Center for Biotechnology Information Expression Omnibus (GEO, http://www.ncbi.nlm.nih.gov/geo/), and are accessible through GEO series accession number GSE23456.

Effect of forced expression of *SOX17* on human and mouse gastric tumor cells. The role of Sox17 on the expression of CD133 was examined by transfecting human and mouse gastric tumor cells with a plasmid encoding human *SOX17* under the control of cytomegalovirus promoter. Informed consent was obtained from the patient and the study was approved by the appropriate institutional review committee. Human *SOX17* was cloned by reverse transcription-PCR (RT-PCR) from cDNA derived from the patient's normal gastric mucosa (forward primer, GTTCGGATCCGCCATGAGCAGCCCGGATGCG; reverse primer, ATGTGAATTCCACGTCAGGATAGTTGCAGTA), and its sequence was confirmed by direct sequencing of PCR products. To generate human *SOX17* expression constructs, the entire encoding region of its cDNA was subcloned in frame into the pcDNA4/myc-His A vector (Invitrogen, Carlsbad, CA, USA) via BamHII and EcoRI sites. MKN45, MKN74 and GIF-5 cells were transfected with human *SOX17*-expressing pcSOX17-myc/pcDNA4 (2 μg/well) using a Neon Transfection System (Invitrogen). Two days after transfection, cells were harvested and their gene expression was investigated by real-time PCR.

Effect of suppression of *Sox17* expression on gastric tumor cells. The effect of suppression of mouse *Sox17* expression was examined using small interfering RNA (siRNA) against mouse *Sox17*. GIF-5 cells were transfected with siSox17-1: CGCACGGAAUUCGAACAGUAU, or siSox17-2: CAGUCUCGGACUAUGCAGUGU (Sigma-Aldrich Japan, Tokyo, Japan; final concentration, 50 nM), using the Neon Transfection System. Negative control siRNA (Applied Biosystems, Carlsbad, CA, USA) was used as a control.

Real-time PCR. RNA was extracted from cell pellets using Trizol (Invitrogen), and was reverse transcribed with SuperScript III (Invitrogen). Real-time PCR was performed using a LightCycler480 (Roche Applied Science, Indianapolis, IN, USA). The expression was normalized to the human *GAPDH*/mouse *Gapdh* expression level, and samples transfected with empty vectors or control siRNA were used as controls. The primers used were as follows: human *GAPDH* (forward, TGCACCACCAACTGCTTAGC; reverse, GGCATGGACTGTGGTCATGAG), human *PROMININ-1* (forward, TGGATGCAGAACTTGACAACGT; reverse, ATACCTGCTACGACAGTCGTGGT), human *SOX17* (forward, GTGGACCGCACGGAATTTG; reverse, GAGGCCCATCTCAGGCTTG), mouse *Gapdh* (forward, TGAAGCAGGCATCTGAGGG; reverse, CGAAGGTGGAAGAGTGGGAG), mouse *Prominin-1* (forward, GTTGAGACTGTGCCCATGAAA; reverse, GACGGGCTTGTCATAACAGGA), and mouse *Sox17* (forward, GATGCGGGATACGCCAGTG; reverse, CCACCACCTCGCCTTTCAC).

Statistical analysis. Some results were statistically analyzed by means of the Chi-squared test or Mann–Whitney's *U*-test, and differences with $P < 0.05$ were considered significant.

Results

Expression of CD133 is related to the morphogenetic potency of gastric epithelial cells. We first examined the expression of CD133 by *Runx3−/−p53−/−* gastric epithelial cells by FACS. GIF-3 cells did not express CD133 and formed poorly differentiated tumors, while GIF-14 cells strongly expressed it and formed well-differentiated tumors (Fig. 1). Interestingly, CD133(+) and CD133(−) populations co-existed among GIF-5 and GIF-11 cells, both of which formed glandular structures *in vivo* and *in vitro*.[13,14] We have established several *Runx3+/+p53−/−* mouse gastric epithelial cell lines, which are not tumorigenic, but form glandular structures when combined with collagen gel *in vitro*.[14] FACS analysis showed that all these cells strongly expressed CD133 (data not shown). We have also established the *Runx3+/−p53−/−* GIF-2 cell line, which was tumorigenic when its passage number was over 40. GIF-2 cells did not express CD133, and formed poorly differentiated tumors (Fig. S1). We also examined the expression of CD133 by human gastric tumor cell lines and found that both MKN45 and MKN74 cells did not express CD133 while KATO-III cells

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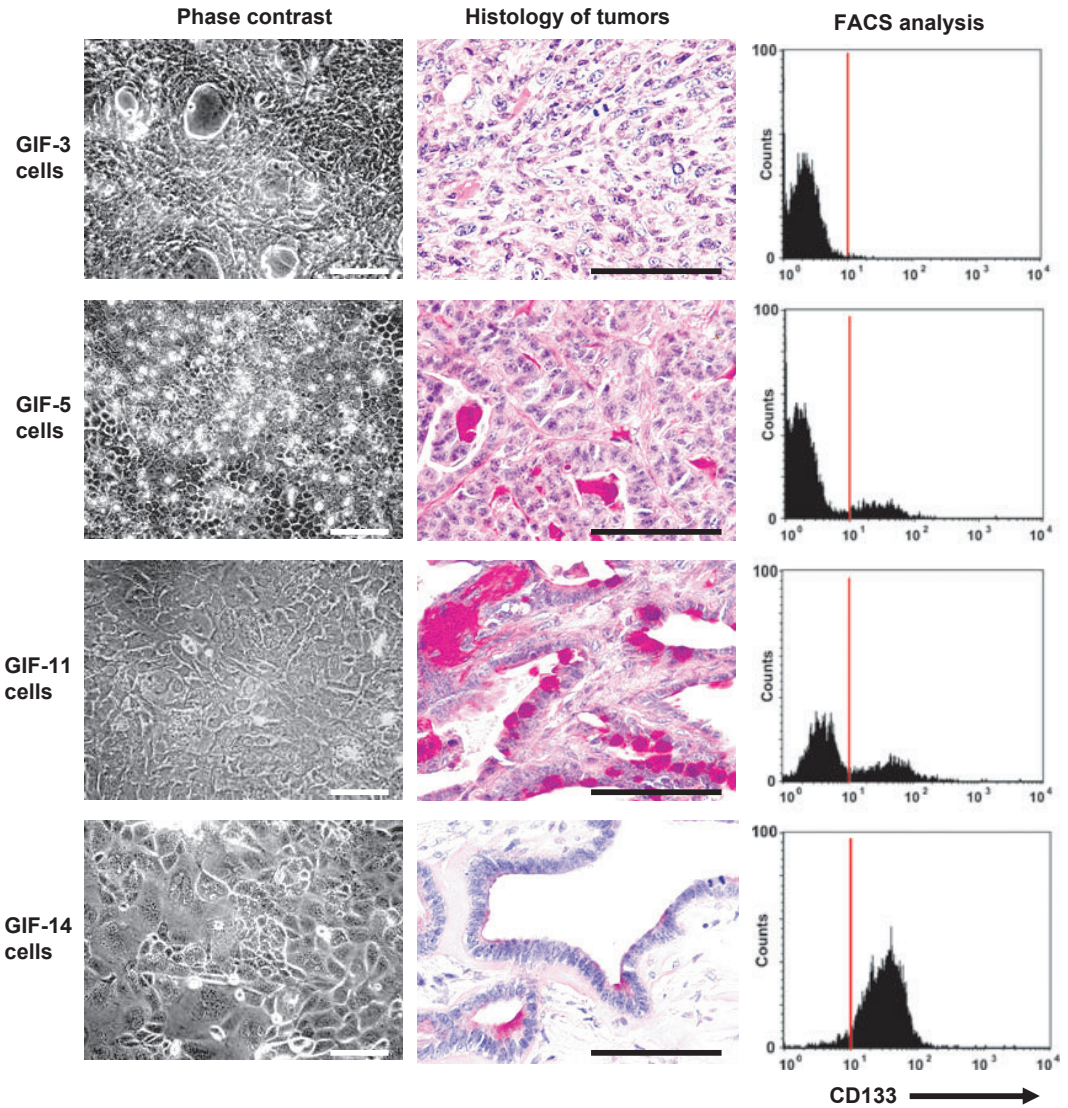

Fig. 1. Characteristics of *Runx3−/−p53−/−* mouse gastric epithelial cells. Phase contrast micrographs, light micrographs of tumors formed in nude mice stained with periodic acid-Schiff-hematoxylin, and FACS analysis on the expressions of CD133 of GIF-3, GIF-5, GIF-11 and GIF-14 cells are shown. Bars, 100 μm.

did, as previously reported.[31,32] When subcutaneously injected into nude mice, the MKN45 and MKN74 cells formed poorly differentiated tumors while the KATO-III cells formed well-differentiated tumors (Fig. S1).

The relationship between CD133 expression and the tumor type formed on subcutaneous injection of cells into nude mice (or morphogenetic potency of the cells *in vitro* in the case of non-tumorigenic cells) is summarized in Table 1. The CD133(−) cell lines formed poorly differentiated tumors in four out of four cases, while the CD133(+) cell lines formed well-differentiated tumors (or glandular structures in the case of the non-tumorigenic cells) in six out of six cases, indicating a significant relationship between the expression of CD133 and the formation of glandular structures ($P = 0.011$). Thus, we concluded that the expression of CD133 is deeply related to the morphogenetic potency of gastric epithelial cells.

CD133(+) cells retain characteristics of mother cells for both GIF-5 and GIF-11 cells. We previously reported that GIF-5 and GIF-11 cells formed glandular structures both *in vivo* and *in vitro*.[13,14] It is interesting that both cell lines contained CD133(+) and CD133(−) populations (Fig. 1). Thus, we sorted cells by FACS, cultured the sorted cells independently and examined the cell surface antigen profiles of their descendents. The proportion of CD133(+) and CD133(−) cells was not significantly changed when CD133(+) GIF-5 cells were cultured (the proportion of CD133[+] cells was 15.3 ± 10.0% and 25.4 ± 13.8% in parental GIF-5 and CD133[+] GIF-5 lines, respectively), but when CD133(−) GIF-5 cells were cultured, the proportion of CD133(+) cells was significantly reduced to almost zero (0.02 ± 0.04%; $P < 0.01$ compared with parental GIF-5 cells), indicating that CD133(+) cells formed both CD133(+) and CD133(−) cells, but that CD133(−) cells formed only CD133(−) cells in GIF-5 cells. The same conclusion was obtained with GIF-11 cells (the proportion of CD133[+] cells was 13.5 ± 7.8% and 41.5 ± 26.2% in parental GIF-11 and CD133[+] GIF-11 lines, respectively, but when CD133[−] GIF-11 cells were cultured, the proportion of CD133[+] cells was significantly [$P < 0.05$] reduced to 4.0 ± 2.8%) (Fig. 2). These results indicate that CD133(+) cells retained the characteristics of the mother cells for both GIF-5 and GIF-11 cells.

CD133(+) cells formed glandular structures in GIF-5 and GIF-11 cells while CD133(–) cells did not. Next we compared the characteristics of CD133(+) and CD133(−) cells for GIF-5 and GIF-11 cells. We could not find any difference between their morphologies or their growth *in vitro* (data not shown). The only difference we could find between them was a difference in their morphogenetic potency. CD133(+) cells formed differentiated-type tumors with glandular structures, as in the case of the parental cells, while CD133(−) cells formed poorly differentiated-type tumors with few glandular structures when GIF-5 cells were injected into nude mice (Fig. 3). Consistent with this, when GIF-11 cells were cultured with Matrigel *in vitro*, CD133(+) cells formed glandular structures, as in the case of the parental cells, but CD133(−) cells rarely formed glandular structures (Fig. 3). These results suggest that expression of CD133 is related to the morphogenetic potency of the gastric epithelial cells. Considering that some poorly differentiated-type cells are derived from well-differentiated types during gastric cancer progression, these results indicate that loss of CD133 and progression of gastric tumors might be controlled through a common mechanism.

Table 1. Relationship between expression of CD133 and tissue types formed by mouse and human gastric epithelial cell lines *in vivo* or *in vitro*

Mouse cell lines		
Tissue type (histology)	CD133-positive cells	CD133-negative cells
Glandular (well-differentiated)	GIF-5, GIF-7, GIF-9, GIF-11, GIF-14	
Non-glandular (poorly differentiated)		GIF-2, GIF-3
Human cell lines		
Tissue type (histology)	CD133-positive cells	CD133-negative cells
Glandular (well-differentiated)	KATO-III	
Non-glandular (poorly differentiated)		MKN45, MKN74
Total number of cell lines		
Tissue type (histology)	CD133-positive cells	CD133-negative cells
Glandular (well-differentiated)	6	0
Non-glandular (poorly differentiated)	0	4

Mann–Whitney's *U*-test of a 2 × 2 contingency table indicates that there is a significant relationship between expression of CD133 and formation of glandular structures ($P = 0.011$).

CD133 expression in human gastric cancers. Next we examined whether loss of CD133 is related to progression of gastric tumors in humans. For the analysis, expression of CD133 was immunohistochemically examined in human gastric specimens. We found that CD133 was expressed only weakly by normal gastric epithelial cells (Fig. 4). In human gastric tumors, some cells expressed CD133 not only in well-differentiated tumors (three out of six cases), but also in mixed-type tumors composed of well-differentiated and poorly differentiated components (three out of four cases). In signet ring cell carcinomas without tubular tissues, CD133-positive cells could not be found (two out of two cases). CD133 was exclusively seen on the cell membranes at the luminal surface of glandular structures in both

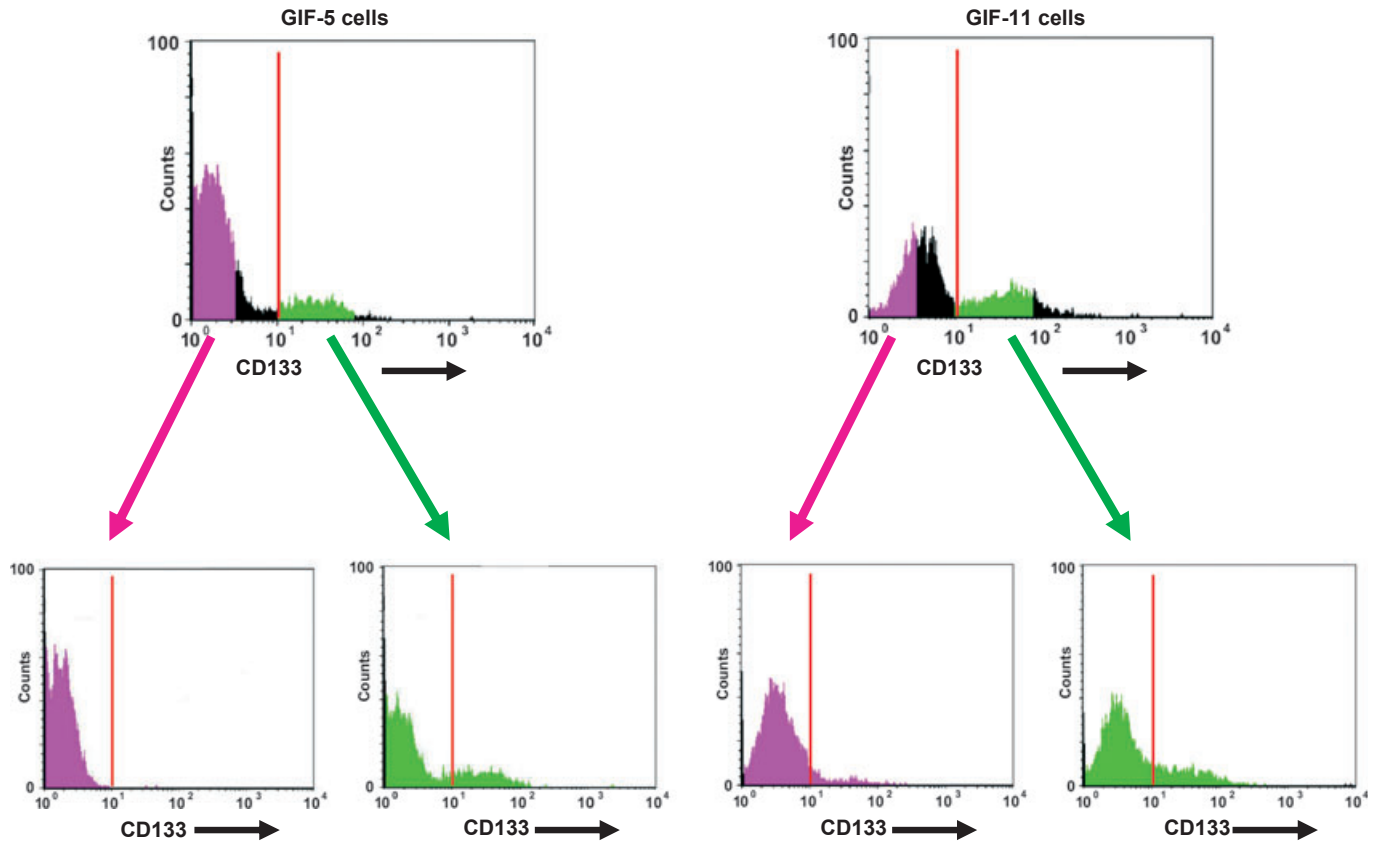

Fig. 2. CD133-positive cells retained the characteristics of the mother cells for both GIF-5 and GIF-11 cells. CD133-positive (green) and CD133-negative (magenta) cells were isolated from GIF-5 (left panel) and GIF-11 (right panel) cells, expanded for 2 weeks and evaluated for CD133 expression. The proportion of CD133-positive cells is similar in the culture of parental and CD133-positive cells, but is significantly decreased to <4% in the culture of CD133-negative cells for both cell lines.

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well-differentiated and a mixture of well-differentiated and poorly differentiated gastric cancers (Fig. 4). In no case was cytoplasmic expression of CD133 found, and diffuse type tumor cells were always negative for CD133. These results with human gastric tumors are consistent with those obtained by experiments with mice, and strongly suggest that loss of expression of CD133 might be related to tumor progression in human gastric cancers.

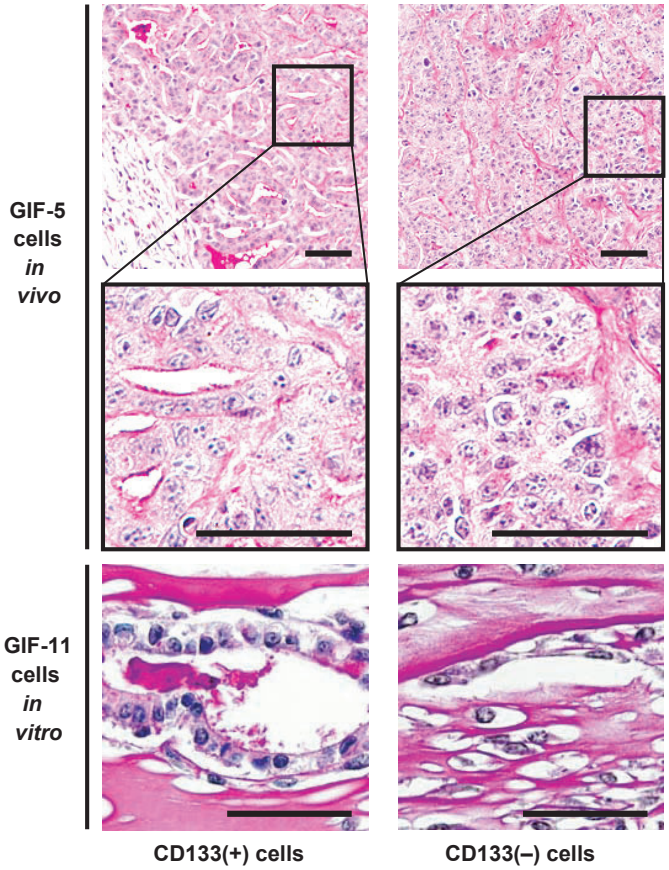

Fig. 3. Characteristics of CD133-positive and CD133-negative cells. These cells were isolated from GIF-5 and GIF-11 cells by FACS, expanded and examined as to their morphogenesis *in vivo* (upper panel) or *in vitro* (lower panel), respectively. Middle panels show enlargements of the boxed areas in the upper panel. Stained with periodic acid-Schiff-hematoxylin. Bars, 50 μm.

CD133(+) and CD133(–) cells exhibit different gene expression profiles. The above results suggest that exploration of the regulatory mechanism for CD133 expression might provide new insights into the mechanism of gastric tumor progression. Thus we explored the mechanism by identifying genes specifically expressed in CD133(+) cells. For this study, we compared the gene expression profiles of CD133(+) and CD133(−) GIF-5 cells because CD133(+) and CD133(−) cells could be clearly separated, and CD133(−) cells never formed CD133(+) cells in GIF-5 cells (Fig. 2). The data summarized in Table 2 show that various important genes, such as those for cadherins 5 and 11, Sox17 and Sox21, nidogen2, and indian and sonic hedgehogs were specifically expressed in CD133(+) cells. These genes were always far more (>10 times) strongly expressed in CD133(+) cells compared with CD133(−) cells. We were especially interested in *Sox17,* because it has been repeatedly reported that Sox17 plays important roles in the control of endodermal development[26,27] and gastrointestinal tumorigenesis.[33]

***Sox17* is specifically expressed in CD133(+) cells and expression of *CD133* is regulated by Sox17.** We first examined whether *Sox17* was specifically expressed in CD133(+) GIF-5 cells by RT-PCR. We found that its expression level was approximately 20–30-times greater in CD133(+) cells than in CD133(−) cells (data not shown). We then examined whether *CD133* expression could be induced in CD133(−) GIF-5 cells through forced expression of *SOX17*. We found that the mouse *CD133* expression levels were significantly increased to approximately three times the control level when human *SOX17*-expressing plasmids were introduced into CD133(−) GIF-5 cells (Fig. 5A). Many permanent human gastric cancer cell lines have been reported not to express CD133.[32] Thus we examined whether *CD133* expression could be induced when human *SOX17*-expressing plasmids were introduced into MKN45 and MKN74 cells, which did not express CD133. Real-time PCR analysis showed that expression of *CD133* was increased to approximately four times in MKN45 cells, and two times in MKN74 cells on introduction of the human *SOX17*-expressing plasmid (Fig. 5B,C). These results suggest that *CD133* expression could be induced by SOX17.

If Sox17 is directly involved in the control of *CD133* expression, reduction of *Sox17* should result in a reduction in expression of *CD133*. We examined this possibility by using siRNA against mouse *Sox17*. First we examined changes in the level of mouse *Sox17* when these siRNA were introduced into *Sox17*- and *CD133*-expressing GIF-5 cells. As shown in Figure 5(D),

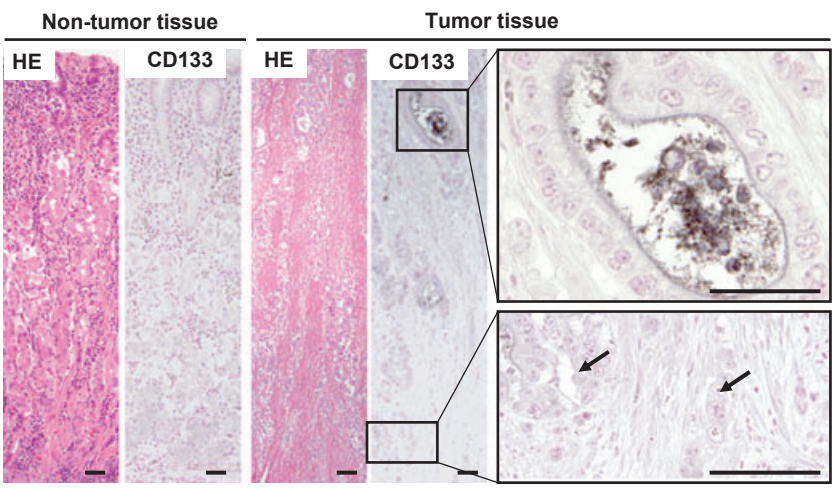

Fig. 4. Expression of CD133 protein in human gastric tissues. Adjacent sections were stained with anti-CD133 antibody (CD133) or hematoxylin–eosin (HE). CD133 was only weakly expressed by non-tumor tissues (left panel), while some glandular tumor tissues expressed CD133 at the luminal surface (upper right panel), but CD133 expression was never found in non-glandular tumor cells (arrows) at the front of invasion (lower right panel). Bars, 5 μm.

the mouse *Sox17* expression levels were significantly reduced by siRNA. Next we examined changes in the level of *CD133* in siRNA-treated cells. As shown in Figure 5(E), the levels were significantly reduced by treatment with siRNA for *Sox17*. These results strongly suggest that Sox17 regulates the expression of *CD133* in gastric epithelial cells.

Table 2. Genes specifically expressed by CD133-positive GIF-5 cells

Gene name	Entrez Gene ID	Ratio of expression in CD133-negative/ CD133-positive cells (analysis #1, analysis #2)	Description
Prom1	19126	(0.00, 0.00)	Prominin 1
Igfbp5	16011	(0.00, 0.01)	Insulin-like growth factor binding protein 5
Cdh5	12562	(0.01, 0.01)	Cadherin 5
Sox17	20671	(0.02, 0.02)	SRY-box containing gene 17
Nid2	18074	(0.02, 0.02)	Nidogen 2
Ihh	16147	(0.03, 0.04)	Indian hedgehog
Tff3	21786	(0.04, 0.04)	Trefoil factor 3, intestinal
Sox21	223227	(0.05, 0.06)	SRY-box containing gene 21
Bambi	68010	(0.06, 0.09)	BMP and activin membrane-bound inhibitor
Cdh11	12552	(0.07, 0.09)	Cadherin 11
Tff2	21785	(0.07, 0.09)	Trefoil factor 2 (spasmolytic protein 1)
Fst	14313	(0.07, 0.10)	Follistatin
Shh	20423	(0.09, 0.11)	Sonic hedgehog
Pax7	18509	(0.09, 0.11)	Paired box gene 7

Relationship between expressions of CD133 and SOX17. We next examined whether there is a close relationship between expressions of CD133 and SOX17 in human gastric tissues. In normal human gastric tissues, SOX17 was expressed by parietal and endothelial cells (Fig. 6). As shown in Figure 4, CD133 was only weakly expressed by normal human gastric tissues including parietal cells, indicating that expression of CD133 was not strongly related to that of SOX17.

Using human gastric tumor tissues we immunohistochemically compared expression of CD133 with that of SOX17, and found that gastric tumor cells expressed CD133 even when they expressed SOX17 weakly (Fig. 6, center panel), and that some tumor cells did not express CD133 even when they expressed SOX17 (Fig. 6, right panel). These results suggest that a strong interrelationship could not be found between the two in human gastric tumor tissues using the current immunohistochemical method.

Discussion

In the present study, we examined the roles of CD133 in the growth and differentiation of gastric epithelial cells. We could not obtain any evidence indicating that CD133 expression is related to their growth, but we obtained evidence suggesting that it is involved in gastric epithelial morphogenesis. As shown in Table 1, CD133 expression was significantly correlated to the formation of glandular structures. Moreover, we found that in two cell lines in which CD133(+) and CD133(−) cells co-existed, CD133(+) cells formed glandular structures while CD133(−) cells did not, both *in vivo* and *in vitro*. These results strongly suggest that CD133 expression and gastric epithelial morphogenesis are controlled through a common mechanism. It

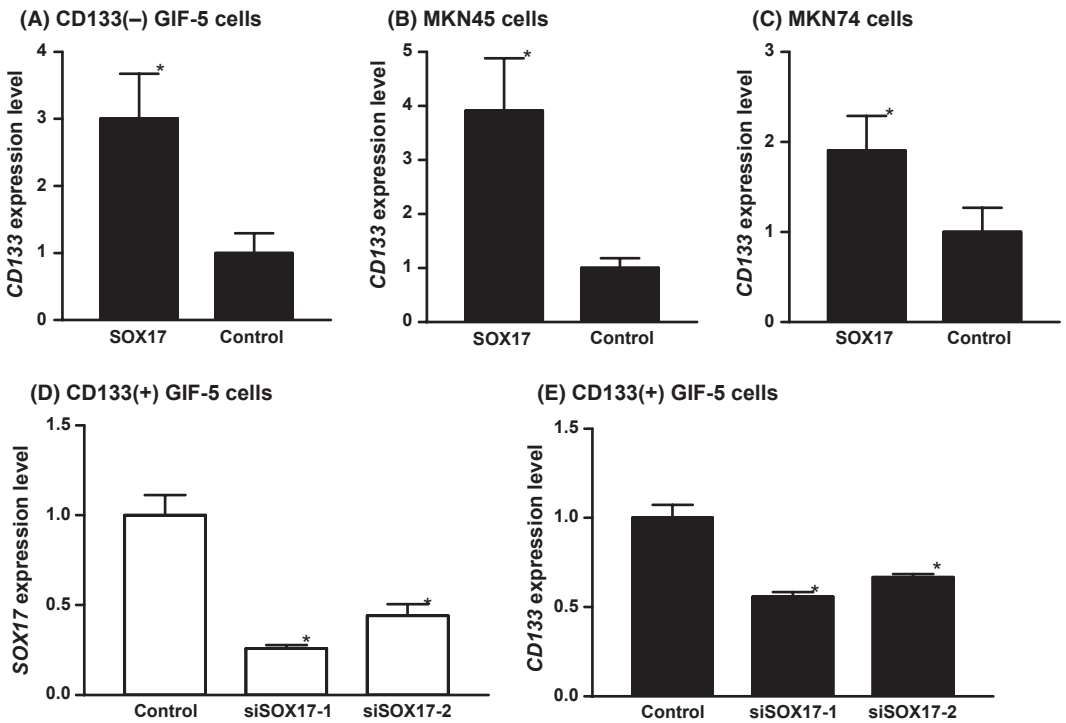

Fig. 5. Expression of *CD133* is regulated by Sox17. CD133-negative GIF-5 (A), MKN45 (B) and MKN74 cells (C) were transfected with a *SOX17*-expressing plasmid, and their *CD133* expression levels were examined. CD133-positive GIF-5 cells were transfected with siRNA for Sox17, and then the expression levels of *Sox17* (D) and *CD133* (E) were determined. *Indicates a significant ($P < 0.05$) difference from controls.

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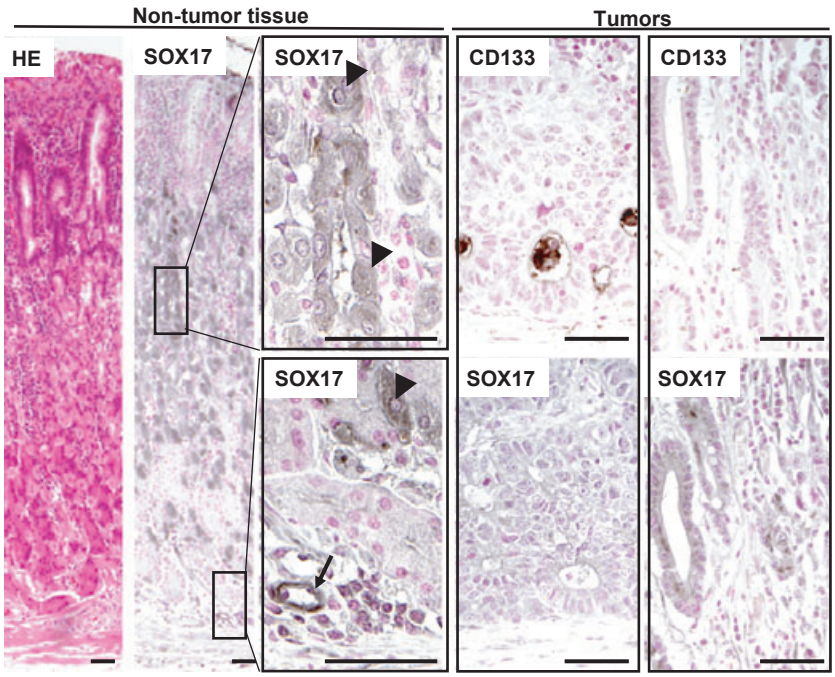

Fig. 6. Expressions of CD133 and SOX17 proteins in human tumor and non-tumor tissues. Adjacent sections were stained with anti-SOX17 (SOX17) or anti-CD133 (CD133) antibodies, or hematoxylin–eosin (HE). In a normal human gastric tissue (left panel), SOX17 was expressed by parietal (arrowheads) and endothelial cells (arrow). In human gastric cancers, tumor cells expressed CD133 even when they expressed SOX17 weakly (center panel), and some tumor cells did not express CD133 even when they expressed SOX17 (right panel). Bars, 5 μm.

has been reported that most human gastric cancer cell lines do not express CD133,[32] and that the morphological characteristics of human gastric carcinomas gradually decrease during passages *in vitro*.[31] Our results are consistent with these, and suggest that the mechanism underlying epithelial morphogenesis might be elucidated by determining how the expression of CD133 is regulated. Boeg and Prinz[25] reported that CD133 expression was reduced in advanced stages of gastric cancer. This might be explained by our finding that CD133 is expressed on well-differentiated-type gastric tumor cells, but not on poorly differentiated-type tumor cells, which are usually found in advanced stages. We found that CD133 was exclusively expressed by glandular cells but not by diffuse-type cells in human gastric cancers (Fig. 4). Similar results have been reported in colorectal tumors,[29,34] ductal adenocarcinomas of the pancreas[35] and esophageal adenocarcinomas,[36] although Zhao *et al.*[37] reported that CD133 was also expressed by cells at invasive depth.

In the present investigation, we found that CD133(+) cells are the mother cells for both CD133(+) and CD133(−) cells, and that CD133(−) cells did not form glandular structures in the absence of CD133(+) cells for both GIF-5 and GIF-11 cells. When GIF-5 or GIF-11 cells were subcutaneously injected into nude mice they formed glandular structures in spite of that CD133(−) cells comprised the major and CD133(+) cells the minor population, indicating that CD133(+) cells induced glandular structure formation by CD133(−) cells. It is possible that CD133(+) cells secrete humoral factors and/or extracellular matrix, which induce glandular structure formation by CD133(−) cells. Elucidation of the mechanism is an important task for the future.

In cultures of GIF-5 and GIF-11 cells, CD133(+) cells formed both CD133(+) and CD133(−) cells, indicating that CD133 expression is easily lost during *in vitro* growth of these cells. If loss of CD133 expression is related to the loss of the morphogenetic ability of the cells, loss of CD133 expression might be observed during the progression of gastric cancers when poorly differentiated-type tumors are formed from well-differentiated types. The mechanism of progression has not been fully elucidated, although the methylation of tumor suppressor genes,[38] secretion of growth factors,[39] epithelial–mesenchymal transition,[40] and fibroblast-derived proteases[41] have been demonstrated to be involved in it. Thus, GIF-5 and GIF-11 cells might be good models for studying the mechanism of gastric tumor progression.

CD133 is widely used for the identification of cancer stem cells in various tumors, but its physiological significance is not fully understood, partly because *CD133*-knockout mice can survive without major defects, although progressive degeneration of mature photoreceptors has been found in these mice.[42] It is probable that expression of CD133 is not directly related to the function of stem cells, but that its expression is controlled through a mechanism that is related to the function of stem cells. Thus, it would be important to elucidate the mechanism regulating the expression of CD133, because it might function in the regulation of the identity of stem cells. Our system involving GIF-5 cells would be useful for future study to identify factors regulating CD133 expression.

To identify the factors, we compared the gene expression profiles of CD133(+) and CD133(−) cells and found that Sox17 was a candidate factor controlling expression of CD133. Forced expression of *SOX17* in CD133(−) cells and reduction of *SOX17* in CD133(+) cells showed that SOX17 regulated *CD133* expression in both mouse and human gastric epithelial cells. Thus, we concluded that Sox17 is a key transcription factor controlling CD133 expression. It is probable that loss of Sox17 induces loss of CD133, which directly/indirectly results in the development of gastric tumors into more aggressive poorly differentiated types. In the present study, we immunohistochemically examined the relationship between expression of CD133 and that of SOX17 in human gastric tumors, but a strong correlation could not be found between them. It is possible that SOX17 is diffused during immunohistochemical procedures because it was found not only in the nuclei but also in the cytoplasm in the present study (Fig. 6). Better results might be obtained by improving the procedures. It is also possible that Sox17 functions in collaboration with other factors to regulate CD133 expression. It is necessary to identify these factors and to elucidate the mechanism to fully understand how gastric epithelial morphogenesis is controlled.

The function of SOX17 in gastric carcinogenesis is not fully understood. Katoh[43] reported that *SOX17* was more preferentially expressed in the esophagus, stomach and small intestine than in the colon and rectum in normal gastrointestinal tract, and that it was almost undetectable in human cancer cell lines and also in 66 cases of human primary tumors derived from various tissues. Consistent with this, a tumor-suppressive role of SOX17 has been reported in colon carcinoma cells[44] and hepatocellular carcinomas.[45] Zhang *et al.*[46] reported that *SOX17* silencing due to promoter hypermethylation was an early event during tumorigenesis in colorectal cancer. We also found that *SOX17* is not expressed in most human gastric tumor cell lines including MKN45 and MKN74, and that this might be caused by promoter hypermethylation because its expression was significantly increased by treatment with 5-aza 2′-deoxycytidine in these cells (Shimada S., Fukamachi H. and Yuasa Y., unpublished data).

Recently, Du *et al.*[33] showed that Sox17 played a tumor suppressive role through suppression of Wnt signaling in mouse gastric carcinogenesis. They also suggested that Sox17 protected early tumors from malignant progression at an early stage of tumorigenesis, and that downregulation of Sox17 contributed to malignant progression through promotion of Wnt activity. These results are consistent with ours and suggest that loss of Sox17 induces gastric tumor progression through, at least in part, the transformation of well-differentiated-type tumors into poorly differentiated types.

In the present study, we could not obtain direct evidence that Sox17 regulates gastric epithelial morphogenesis, because we could not induce glandular structure formation by MKN45 or MKN74 cells, even when expression of *SOX17* and *CD133* was significantly elevated (data not shown). It is probable that not only *SOX17* expression but also the expression of other genes might be necessary for the cells to form glandular structures. Gland-forming GIF-5 cells specifically expressed various genes that are related to cell attachment and extracellular matrix deposition (Table 2). Combinational expression of these genes might be necessary to induce glandular structure formation. Further studies are needed to elucidate the mechanism by which gastric epithelial morphogenesis is regulated.

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Disclosure Statement

The authors have no potential conflicts of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Fig. S1. Characteristics of KATO-III, MKN45, MKN74 and GIF-2 cells *in vitro* and in nude mice.

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