In vitro differentiation of *Runx3−/−p53−/−* gastric epithelial cells into intestinal type cells

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We have reported that a lack of RUNX3 function is causally associated with gastric carcinogenesis. We have also presented evidence that loss of *Runx3* may be related to the genesis of intestinal metaplasia because expression of *RUNX3* is reduced in some intestinal metaplasias, and some *Runx3−/−p53−/−* gastric epithelial cells differentiate into intestinal type cells *in vivo*. Recently several reports have indicated that blood cells play important roles in the gastric carcinogenesis. In the present study, we therefore examined whether *Runx3−/−p53−/−* gastric epithelial cells differentiate autonomously into intestinal type cells, or whether the presence of other cells is necessary for the differentiation *in vitro*. When *Runx3−/−p53−/−* gastric epithelial cells were cultured with collagen gels, they did not exhibit any morphogenesis and differentiated poorly. When cultured with fetal mouse gastric mesenchymes, the cells formed glandular structures and differentiated into surface mucous cells, but differentiation of intestinal type cells was never observed. When cultured with Matrigel, the cells formed glandular structures, and some cells differentiated into intestinal type cells *in vitro*. Reverse transcription–polymerase chain reaction analysis showed that the cells expressed stomach-specific genes, and their levels decreased gradually during the culture. The cells expressed some intestine-specific genes weakly at the start of culture, and their levels were increased with time in culture. We therefore conclude that *Runx3−/−p53−/−* gastric epithelial cells differentiate into intestinal type cells in combination with Matrigel in the absence of other cell types. Extracellular matrix, not blood cells, may play a role in the genesis of intestinal metaplasia. (*Cancer Sci* 2008; 99: 671–676)

Gastric cancer is one of the leading causes of cancer deaths worldwide.[1] Gastric adenocarcinoma of the intestinal type is assumed to be formed in a stepwise fashion from chronic gastritis to atrophic gastritis, followed by intestinal metaplasia, leading to dysplasia and carcinoma.[2] Intestinal metaplasia in the stomach represents the conversion of gastric type mucosa to a mucosa that closely resembles the intestinal one,[3] and has been considered to be precancerous for gastric carcinogenesis,[4] although there is controversy over whether intestinal metaplasia is a paraneoplastic change in gastric carcinogenesis.[5] Several environmental factors, such as *Helicobacter pylori* infection,[6] carcinogen treatment,[7] and X-ray irradiation,[8] have been shown to induce intestinal metaplasia, but little is known about the molecular mechanism how gastric epithelial cells differentiate into intestinal type cells, and whether these factors are essential for the genesis of intestinal metaplasia. Recently, expression of *Cdx2* has been demonstrated to be sufficient to induce intestinal metaplasias in the stomach in transgenic mice,[9,10] but it remains to be solved how Cdx2 induces differentiation of intestinal type cells.

We have 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.[11] We have also found that expression of *RUNX3* is reduced in some intestinal metaplasias in human stomachs,[11] and some *Runx3−/−p53−/−* gastric epithelial cells differentiate into intestinal type cells *in vivo*,[12] suggesting that a lack of RUNX3 function is also related to the genesis of intestinal metaplasia. Recently, several reports have indicated that blood cells play important roles in gastric carcinogenesis. Houghton *et al.* showed that bone marrow-derived cells repopulated the gastric mucosa, and contributed over time to metaplasia, dysplasia, and gastric cancer in bone marrow-transplanted mice.[13] Brenner *et al.* reported that loss of Runx3 function induced leukocyte infiltration in the gut, and suggested that this might induce a progressive hyperplasia of the gastric mucosa.[14]

To examine whether *Runx3−/−p53−/−* gastric epithelial cells differentiate autonomously into intestinal type cells, or whether other cells or factors are necessary for the differentiation, we cultured the cells in combination with various mesenchymal components, and their differentiation *in vitro* was investigated histochemically and molecular biologically. The results indicate that *Runx3−/−p53−/−* gastric epithelial cells differentiate into intestinal type cells *in vitro* in the absence of other cell types.

Materials and Methods

Runx3−/−p53−/− gastric epithelial cell lines. Establishment of a GIF-11 *Runx3−/−p53−/−* gastric epithelial cell line has been described in previous papers.[11,12] Briefly, 16.5-day fetal mouse gastric epithelia were separated from attaching mesenchymes using 30 mM EDTA–Hanks' solution, and epithelial tissues from a fetus were cultured separately on collagen gel. After several passages, the cells began to grow rapidly on plastic in DMEM (Sigma-Aldrich, St Louis, MO, USA) supplemented with 10% FBS (Sigma-Aldrich) in a humidified atmosphere of 5% CO_2 in air. The cells were subcultured by treatment with trypsin–EDTA. In the present study, GIF-11 cells were used at passage number 40–55.

Organ culture. The differentiation and morphogenesis of the cells *in vitro* was examined by culturing them in combination with various mesenchymal components, as shown in Figure 1. Detailed methods for combination with fetal mouse gastric mesenchymes,[15] rat tail collagen gel,[16] or Matrigel (BD Biosciences, Franklin Lakes, NJ, USA)[17] have been described in previous papers. Briefly, a drop of collagen or Matrigel was placed on membrane filters (HAWP; Millipore, Carrigtwohill, County Cork, Ireland), and 10^4 cells were seeded on it using a

cloning cup. They were cultured overnight, and on the next day (day 1) they were covered with mesenchymes from 16.5-day fetal BALB/c mice (Charles River Japan, Yokohama, Japan) or another drop of collagen gel or Matrigel. Cells were cultured at the air–liquid interface by placing the filter with cells and mesenchymal components on a stainless steel grid, which was placed in a dish. The culture medium was DMEM with 10% FBS, and was changed once a week. After 1–6 weeks, cells with mesenchymal components were processed for histological and molecular biological analyses.

Histological methods. Cells with mesenchymal components were fixed with cold 95% ethyl alcohol, embedded in paraffin, sectioned at 5 μm, and stained with PAS–hematoxylin. Some were also stained with AB–neutral red, or HID–AB to detect sulfated mucosubstances, as described previously.[12]

RT-PCR. Glandular stomach and duodenal tissues were obtained from 3-month-old male mice. Total RNA was extracted from the cells and tissues using Trizol (Invitrogen, Carlsbad, CA, USA), according the manufacturer's instructions. Approximately 1 μg of total RNA from each tissue was reverse transcribed to cDNA using SuperScript II Reverse Transcriptase (Invitrogen), and the resulting cDNA was subjected to 21–36 cycles of PCR. The following oligonucleotide primer sets were used for the PCR reactions (expected product size in parentheses): Mucin1 (700 bp), 5′-GGCTCAGCCACCAGTCCA-3′ (sense) and 5′-TGGGCAGAGGGAGGGAACT-3′ (antisense); Sox2 (465 bp), 5′-GCCCAGTAGACTGCACATGG-3′ (sense) and 5′-GCGAACTCCCTGCGAAGCG-3′ (antisense); IF (600 bp), 5′-GAACTGGTCACCTTCAAGC-3′ (sense) and 5′-AGCAGATCAACCCCTCTCA-3′ (antisense); Mucin2 (339 bp), 5′-ACACTCAGCACACCAACCAA-3′ (sense) and 5′-GGTAATCTCCTCCACCAGCA-3′ (antisense); Cdx2 (300 bp), 5′-AGTCGATACATCACCATCAGGA-3′ (sense) and 5′-AGAGAATTCTCACTGGGTGACAGTGGAGTT-3′ (antisense); SI (376 bp), 5′-TAGCTGCAGATGACAACCAAAC-3′ (sense) and 5′-TCAGGAATTCAAACTGATGGTG-3′ (antisense); GAPDH (415 bp), 5′-GACCACAGTCCATGCCATCAC-3′ (sense) and 5′-GTCCACCACCCTGTTGCTGTA-3′ (antisense). The PCR products were separated by electrophoresis on 2.0% agarose gels in 1× TAE buffer, and detected by ethidium bromide staining. GAPDH mRNA controlled for equal sample loading.

Results

GIF-11 *Runx3⁻ᐟ⁻p53⁻ᐟ⁻* gastric epithelial cells attached closely to each other on Matrigel. GIF-11 *Runx3⁻ᐟ⁻p53⁻ᐟ⁻* gastric epithelial cells proliferated rapidly in cell culture on plastic, with an approximate doubling time of 24–30 h. They did not form a cell sheet on plastic, but individual cells often exhibited a criss-cross pattern or piling up (Fig. 2a). When cultured on collagen gel, GIF-11 cells showed similar morphologies as on plastic, but some cells attached closely to each other to form small cell masses (Fig. 2b). When cultured on Matrigel, almost all GIF-11 cells soon attached very closely to each other to form cell masses where cell boundaries could not be easily found (Fig. 2c).

GIF-11 cells differentiated into both gastric and intestinal type cells in organ culture. When cultured with collagen gel, GIF-11 *Runx3⁻ᐟ⁻p53⁻ᐟ⁻* cells formed a simple cuboidal cell layer on the
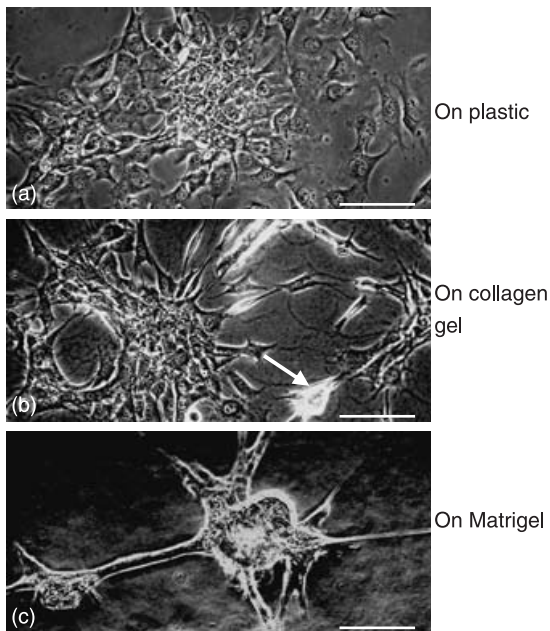
Fig. 2. Phase contrast micrographs of GIF-11 cells cultured on (a) plastic, (b) collagen gel, and (c) Matrigel for 1 day. (b) Some cells on the collagen gel attach closely to each other to form small cell masses (arrow), (c) whereas on Matrigel, much greater cell masses are formed. Scale bars = 100 μm.

surface of the gel after 2 weeks (Fig. 3b), and they became stratified after 3 weeks (Fig. 3c). Some cells migrated into the gel, but none of the cells exhibited morphogenesis, and no differentiation was observed during culture, except that weak

PAS-positive materials were found between the cells (Fig. 3c).

When combined with fetal gastric mesenchymes, GIF-11 cells migrated into the mesenchymal tissue and formed glandular structures after 1 week (Fig. 3d), suggesting that fibroblastic cells are needed for their morphogenesis. The cells exhibited few differentiated characteristics at the start of culture, but after 1–2 weeks, PAS-positive mucus was found on their luminal surface, indicating that the cells differentiated into mucus-secreting cells (Fig. 3e). After 3 weeks, some cells differentiated into surface mucous cells with elongated nuclei and large PAS-positive, AB-negative, and HID-negative mucus droplets in their cytoplasm (Fig. 3f). The cells soon degenerated, and no live cells were found after 4–5 weeks (data not shown). Differentiation of intestinal type cells was never found when GIF-11 cells were cultured with gastric mesenchymes.

When cultured with Matrigel, some GIF-11 cells migrated into the gel to form glandular structures after 1–2 weeks (Fig. 3g,h). The gland-forming cells exhibited polarity, with PAS-positive mucus localized on their apical surface after 3 weeks (Fig. 3i). They did not differentiate into typical surface mucous cells, found in cultures with gastric mesenchymes. Rather, some cells differentiated into intestinal type cells, which were first found after 4 weeks by the presence of goblet cells, with large PAS-positive materials (Fig. 4g). Histochemical analysis showed that these granules were AB⁻ positive (Fig. 4h) and HID⁻ positive (Fig. 4i), indicating that the cells contained sulfated mucins, which are found in intestinal epithelia (Fig. 4d,f) but not in gastric epithelia (Fig. 4c,e). After 6 weeks, most cells contained PAS-positive mucus (Fig. 4j), but small PAS-positive granules were AB⁻ negative and HID⁻ negative, and large PAS-positive granules were AB⁻ positive and HID⁻ positive, indicating that some cells differentiate into gastric type mucus cells whereas others differentiate into intestinal type goblet cells (Fig. 4j–l).
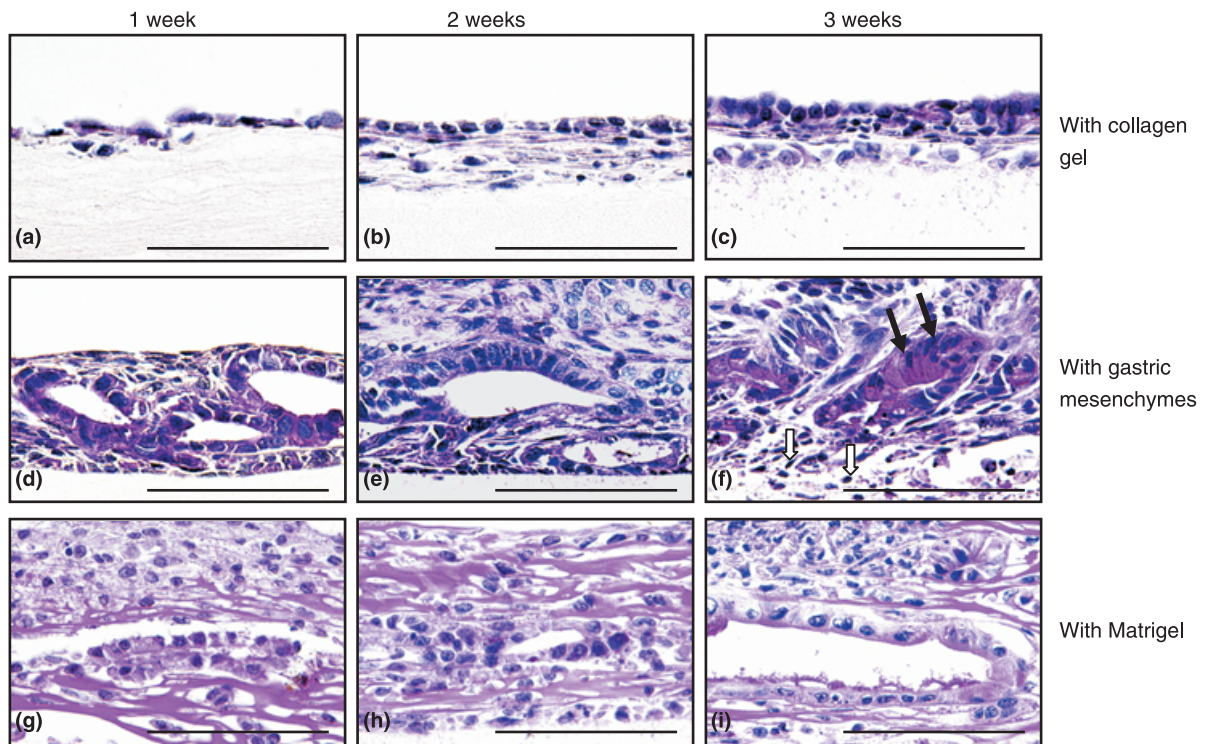

Fig. 3. Morphogenesis and differentiation *in vitro* of GIF-11 cells cultured with (a–c) collagen gels, (d–f) gastric mesenchymes, and (g–i) Matrigel, for (a,d,g) 1, (b,e,h) 2, and (c,f,i) 3 weeks. Sections are stained with periodic acid–Schiff (PAS) and hematoxylin. (f) Closed arrows show surface mucous cells characterized by large PAS-positive materials and elongated nuclei. Some fibroblasts die during long-term culture, and open arrows in (f) show the nuclei of dying cells. No leukocytes can be found in the mesenchyme in organ culture. Scale bars = 100 μm.
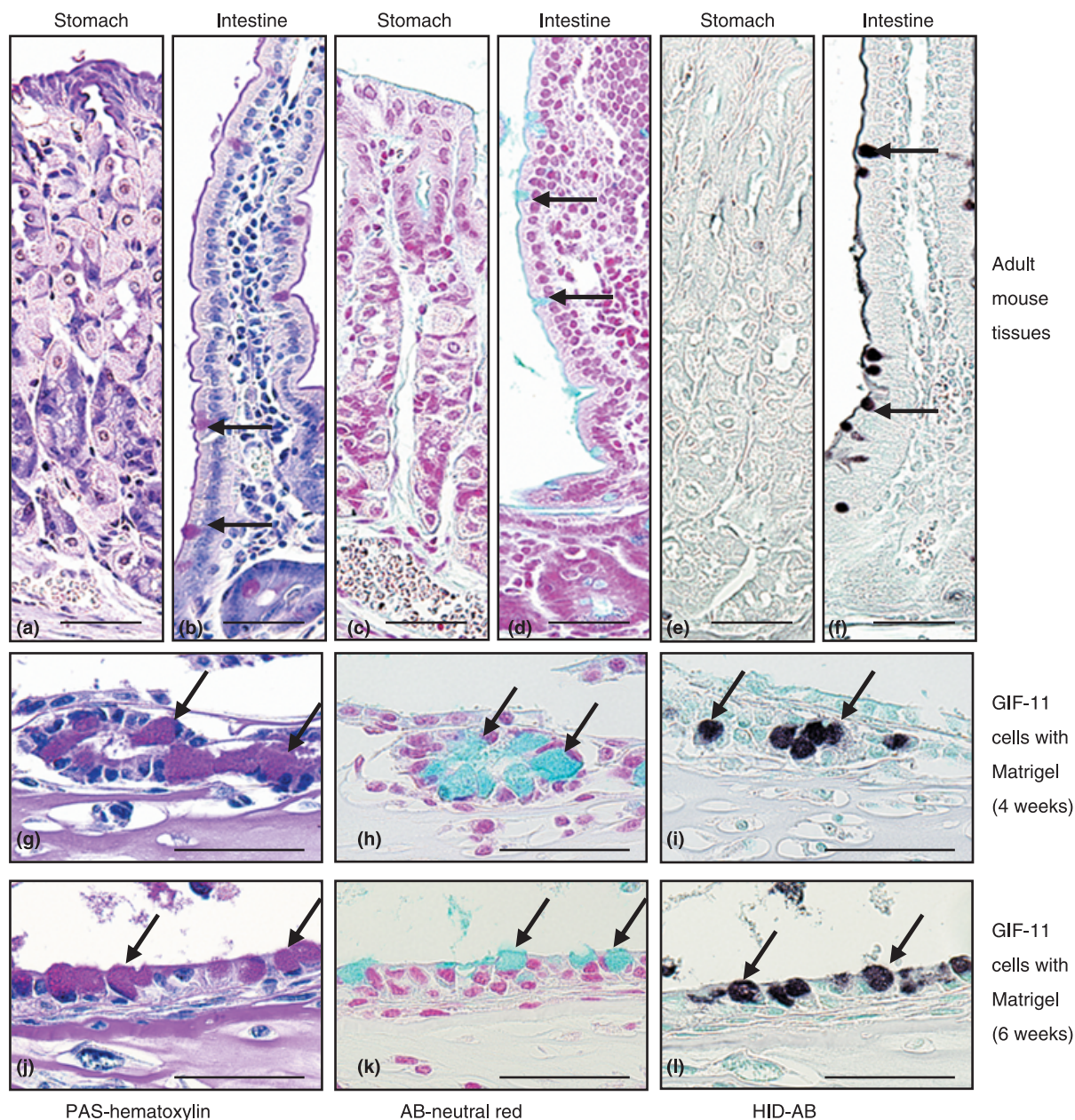
Fig. 4. Differentiation of mucus-containing cells in an adult mouse (a,c,e) stomach and (b,d,f) small intestine, and in GIF-11 cells cultured with Matrigel for (g–i) 4 and (j–l) 6 weeks. Periodic acid–Schiff (PAS) and hematoxylin staining shows that (g,j) some GIF-11 cells contain large PAS-positive mucus droplets (arrows), (b) found specifically in goblet cells in the intestine (arrows). Alcian blue (AB)-neutral red and high iron diamine (HID)-AB staining shows that (h,k) the mucus droplets in GIF-11 cells are AB-positive (arrows) and (i,l) HID-positive (arrows), (d,f) as is the case for mucus in goblet cells, (c,e) whereas mucus in gastric epithelial cells is negative for both stains. Scale bars = 50 μm.

RT-PCR analysis on the differentiation of GIF-11 cells cultured with collagen gel or Matrigel. The differentiation of GIF-11 cells cultured with collagen gel or Matrigel was further examined by analyzing the expression of *Cdx2*, *IF*, *Muc1*, *Muc2*, *SI*, and *Sox2*. Control experiments with gastrointestinal tissues of adult mice showed that *Muc1*, *Sox2*, and *IF* were expressed exclusively by gastric tissues, whereas *Muc2*, *Cdx2*, and *SI* were expressed specifically by intestinal tissues (Fig. 5, left panel), consistent with previous reports.[18] We thus used these six genes as region-specific markers to specify how gastric epithelial cells differentiate into intestinal type cells in culture.

In cell culture on plastic (data not shown) and also when cultured with collagen gel, GIF-11 cells expressed *Muc1* and *Cdx2* strongly at the start of culture when they proliferated rapidly.

When cultured with collagen gel, the cells also expressed *Sox2*, *IF*, and *Muc2*, indicating that the cells exhibit intermediate characteristics between gastric and intestinal epithelial cells. The levels of *Muc1*, *Sox2*, *IF*, and *Muc2* increased gradually during culture, whereas the level of *Cdx2* did not increase (Fig. 5, middle panel). We suppose that GIF-11 cells tend to differentiate into both gastric and intestinal type cells in culture with collagen gel, but that the cells could not differentiate fully, possibly because some essential factors are not contained in the gel.

When cultured with Matrigel, GIF-11 cells expressed *IF*, *Muc1*, and *Sox2*, marker genes for gastric epithelial cells, at the start of culture, indicating that they exhibit characteristics of gastric epithelial cells when combined with Matrigel. They also
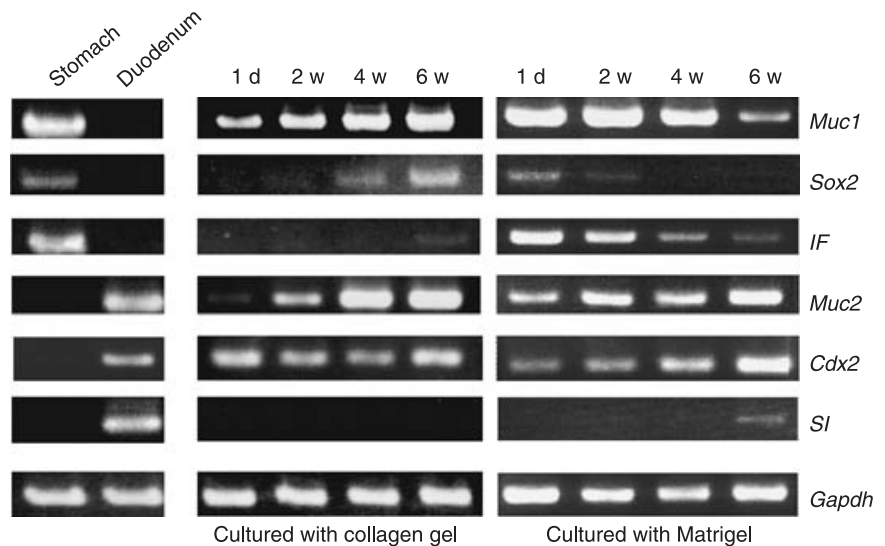
Fig. 5. Reverse transcription–polymerase chain reaction analysis on the differentiation of GIF-11 cells combined with collagen gel (middle panel) or Matrigel (right panel) for 1 day (1 d), 2 weeks (2 w), 4 weeks (4 w), and 6 weeks (6 w). Mucin1 (*Muc1*), *Sox2*, intrinsic factor (*IF*), Mucin2 (*Muc2*), *Cdx2*, and sucrase isomaltase (*SI*) are used as tissue-specific markers for differentiation, as *Muc1*, *Sox2*, and *IF* are expressed explicitly by gastric (stomach) epithelial cells, and *Muc2*, *Cdx2*, and *SI* are expressed specifically by intestinal (duodenum) epithelial cells (left panel). *Gapdh*, glyceraldehyde 3-phosphate dehydrogenase.

expressed *Muc2* strongly and *Cdx2* faintly, indicating that the cells express some intestinal type genes, even when they do not exhibit any morphological characteristics of intestinal type cells. When cultured with Matrigel, the level of gastric type gene expression gradually decreased, whereas that of intestinal type gene expression increased progressively. The expression of *Sox2* decreased greatly after 2 weeks, and it could not be detected after 4 weeks. In contrast, that of *Cdx2* was greatly increased after 4 weeks, and reached a maximum level after 6 weeks. The expression of *SI* could not be detected at the start of culture, and was first noted after 4 weeks when differentiation of intestinal type cells was first noted. Its level was greatly increased after 6 weeks (Fig. 5, right panel). These results indicate that the potency to differentiate into intestinal and gastric type cells gradually increased and decreased, respectively, when the cells were cultured with Matrigel, and that factors contained in Matrigel may facilitate the differentiation of intestinal type cells.

Discussion

In the present study, we found that GIF-11 gastric epithelial cells differentiated poorly in combination with collagen gel, but that they formed glandular structures and differentiated into surface mucous cells when combined with fetal gastric mesenchymes, and that some of them differentiated into intestinal type cells when cultured with Matrigel. The results are consistent with a previous report where *Runx3*−/−*p53*−/− cells did not exhibit any morphogenetic potencies and differentiated poorly when cultured with collagen gel.[12] RT-PCR analysis of GIF-11 cells combined with Matrigel showed that the cells expressed both gastric-specific (*IF*, *Muc1*, and *Sox2*) and intestine-specific (*Muc2* and *Cdx2*) genes at the start of culture, and that the level of gastric type genes was decreased and the level of intestinal type gene expression was increased during culture, resulting in the differentiation of intestinal type cells. The results are consistent with a previous immunohistochemical analysis that showed that intestinal type cells differentiated from GIF-11 cells in nude mice expressed Cdx2.[12] There are several papers reporting differentiation of intestinal type cells in transgenic mouse stomachs,[9,10] but it is not clear from these previous reports whether intestinal type cells transdifferentiated from gastric type cells, or undifferentiated cells residing in the gastric stem cells exhibited intestinal type differentiation in the mouse. The present study clearly showed that GIF-11 cells expressing gastric-specific genes differentiate into both surface mucous cells and intestinal type cells, depending on the culture conditions. To our knowledge, this is the first study showing that intestinal type cells differentiate from gastric type stem-like cells *in vitro*. Tatematsu *et al.* suggested that gastric and intestinal mixed type intestinal metaplasia might be formed by differentiation of stem cells that can produce both gastric and intestinal type cells.[5] Thus, the characteristics of GIF-11 cells are similar to those of the stem cells suggested by Tatematsu *et al.*[5] GIF-11 cells may be useful for further studies to analyze how intestinal metaplasia is generated in the stomach, and how intestinal metaplasia is related to gastric carcinogenesis.

There are several papers suggesting that bone marrow-derived cells or leukocytes play a role in the genesis of gastric hyperplasia and gastric cancer.[13,14] In the present study, we used GIF-11 cells, which were established from gastric epithelial cells of a *Runx3*−/−*p53*−/− mouse fetus using a technique that allows complete separation of epithelial and mesenchymal tissues without cross contamination.[19,20] Thus, the present experiment clearly shows that *Runx3*−/−*p53*−/− gastric epithelial cells differentiate into intestinal type cells in the absence of blood cells. Concerning intestinal metaplasia induced by loss of function of Runx3, bone marrow-derived cells and leukocytes do not seem to play a major part.

In the present investigation, we found that GIF-11 *Runx3*−/−*p53*−/− cells expressed both gastric type and intestinal type genes at the start of culture when combined with collagen gel or Matrigel. The result indicates that the cells retain intermediate characteristics between gastric and intestinal type cells. We have previously found that the expression of *RUNX3* is downregulated in some cases of intestinal metaplasia,[11] and that differentiation of intestinal type cells is found only in *Runx3*−/−*p53*−/−, but not *p53*−/− gastric epithelial cells.[12] We also observed ectopic expression of *Cdx2* in the stomach epithelium in *Runx3*−/− mice while it was never expressed in wild-type stomachs (K. Ito *et al.*, unpublished observation, 2004). All of these results suggest that Runx3 plays an important role in the control of intestinal type genes in gastric epithelial cells. Taniuchi *et al.* have shown that the Runx protein is important for lineage specification in developing lymphocytes.[21] Runx3 may also play an important role in the specification of gastric epithelial cells, and loss of Runx3 may destabilize the differentiation of gastric epithelial cells. We speculate that during culture with Matrigel, expression of *Cdx2* in the destablized cells increased gradually by an unknown mechanism, and its level rose above a threshold to induce differentiation of intestinal type cells after 4 weeks.

Silberg *et al*. found that ectopic expression of *Cdx2* in the gastric epithelium is sufficient to cause transdifferentiation of gastric mucosa into intestinal type cells.[9] They also found that *SI* was not activated in the transgenic mouse stomach, and speculated that other factors may be necessary for the full differentiation of intestinal epithelial cells. Mutoh *et al*. reported that all of the gastric mucosal cells except enterochromaffin-like cells were completely replaced by intestinal type cells, including goblet cells and absorptive cells, in another transgenic mouse strain where *Cdx2* was expressed in parietal cells under the control of the *H+/K+-ATPase* promoter.[10] In this case, parietal cells disappeared after approximately 6 weeks, and the pH in the stomach increased from 2 to more than 7. Watanabe *et al*. repeatedly suggested that elevation of pH in the gastric juice due to the loss of parietal cells in the fundic mucosa induced the development of intestinal metaplasia in the rat.[22,23] Therefore, differentiation of intestinal type cells may be induced not by the expression of *Cdx2*, but by the loss of parietal cells in the transgenic stomach reported by Mutoh *et al*.[10] We found in the present investigation that GIF-11 *Runx3*−/−*p53*−/− cells expressed *SI* when cultured with Matrigel. Thus, not only the expression of *Cdx2* but also the loss of *Runx3* may be essential for the differentiation of intestinal type cells.

In the present study, we found that GIF-11 gastric epithelial cells differentiated into surface mucous cells when cultured with gastric mesenchymes, but that they differentiated into intestinal type cells when combined with Matrigel. It remains to be solved why intestinal type differentiation could not be found in culture with gastric mesenchymes. It is possible that mesenchymes could not survive long culture periods up to 4 weeks when the differentiation of intestinal type cells was first noted, and that epithelial cells died before the differentiation of intestinal type cells in combination with mesenchymes. It is, however, more probable that Matrigel contains factors necessary to induce intestinal type differentiation of GIF-11 cells, and that gastric mesenchymes secrete factors that inhibit intestinal type differentiation and promote gastric type differentiation of the cells. Consistent with this idea, we found that surface mucous cells were differentiated only when GIF-11 cells were combined with gastric mesenchymes but not with Matrigel. Also, Tsukada *et al*.[24] have reported the importance of gastric mesenchymes in inducing gastric epithelial differentiation in rat fetuses. Matrigel has been reported to contain various extracellular matrix and growth factors, including laminin, type IV collagen, and insulin-like growth factor-1.[25] It is a problem for the future to identify factors that facilitate the differentiation of GIF-11 cells into intestinal type cells.

Intestinal metaplasia is considered to be preneoplastic for gastric carcinogenesis. It is possible that metaplastic tissues are reverted to normal tissues to inhibit carcinogenesis if the deregulated system is normalized. We have previously reported that expression of *RUNX3* is often silenced by hypermethylation of its promoter region in human gastric tissues.[11] If the loss of Runx3 function plays an important role in the genesis of intestinal metaplasia, differentiation of metaplastic tissues into normal gastric tissues may be induced by reactivating the silenced gene by using reagents such as demethylating agents or histone deacetylation inhibitors. Further investigations are necessary to identify such chemicals that induce redifferentiation of metaplastic tissues. Our *in vitro* culture system would be very useful for these purposes.

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