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Runx3$^{-/-}$ gastric epithelial cells differentiate into intestinal type cells

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Abstract

We have previously reported that Runx3, a runt domain transcription factor, is a major growth regulator of gastric epithelial cells, that a lack of RUNX3 function is causally related to the genesis and progression of human gastric cancer, and that expression of RUNX3 is greatly reduced in intestinal metaplasias in human stomachs. Here we examined the differentiation of *Runx3*$^{-/-}$ mouse gastric epithelial cells and found that some cells differentiated into intestinal type cells, which expressed *Cdx2*, a transcription factor that has been shown to induce intestinal metaplasia in transgenic mice. Differentiation of intestinal type cells was not found in culture of *Runx3*$^{+/+}$ gastric epithelial cells. These results suggest that gastric epithelial cells can differentiate into intestinal type cells, probably due to expression of *Cdx2* in them when the function of Runx3 is impaired. The relationship between loss of function of Runx3, formation of intestinal metaplasia, and gastric cancer was discussed.

Keywords: Intestinal metaplasia; Gastric cancer; Runx3; Cdx2; Transdifferentiation

Intestinal metaplasia (IM) in the stomach represents the conversion of gastric type mucosa to a mucosa that closely resembles the intestinal one. A strong correlation between IM and gastric carcinoma has been documented in humans: populations demonstrating high incidences of gastric carcinoma have high incidences of IM, and histological analysis has revealed microcarcinomas of intestinal type to be surrounded by IM at very high frequencies. Thus, IM has been assumed to be precancerous for gastric carcinogenesis [1,2], but causal relationship between them has not been ascertained.

There is a controversy as to whether IM is a precancerous lesion or not, since it is also possible that: (a) IM causes an appropriate milieu for carcinogenesis, by raising pH of the gastric juice, thus improving growth conditions for bacteria that directly or indirectly induce gastric cancer, or that (b) IM is just a paraneoplastic lesion resulting from the same mutagenic stimuli which induce gastric cancer [3]. IMs accompanying gastric adenocarcinomas have been successfully induced in rats given *N*-methyl-*N*′-nitro-*N*-nitrosoguanidines in their drinking water [4,5]. Using the system, Tatematsu et al. [6,7] have proposed that IM may not be a preneoplastic change for gastric carcinoma, but that IMs and intestinal type tumor cells may appear independently during the carcinogen-induced gastric carcinogenesis, since: (a) adenomatous hyperplasias without intestinal type cells appear first, and those with intestinal type cells occur later, and (b) more than 50% of adenomatous hyperplasias and adenocarcinomas are composed of gastric type cells, and no tumors consisting solely of intestinal type cells were observed. They suggest that gastric and intestinal type cells were produced from the same germ cells because they found both cell types in the same acini in some adenomatous hyperplasias and adenocarcinomas.

It is also a problem how IM is induced. Do intestinal type cells arise from gastric type cells by transdifferentiation,

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or from undifferentiated multipotent cells residing between gastric type cells? Recently, expression of *Cdx2* has been demonstrated to be sufficient to induce IM in the stomach [8,9], but it remains to be solved which cell is the germ cell for IM, how the expression of *Cdx2* is regulated in the stomach, and whether IM is related to gastric carcinogenesis or not.

RUNX3, a Runt domain transcription factor, is an important target of TGF-β superfamily signaling [10]. We have recently reported that Runx3 is a major growth regulator of gastric epithelial cells, that a lack of RUNX3 function is causally related to the genesis and progression of human gastric cancer, and that expression of RUNX3 is greatly reduced in IMs in human stomachs [11], but the role of Runx3 on the differentiation of gastric epithelial cells has not been fully examined. It is probable that Runx3 plays a role in inducing IM, since development of IM was reported in *TGF-β1*$^{-/-}$ mouse stomachs [12] and TGF-β signal transduction is impaired in the absence of Runx3 [13].

Here we examined the differentiation of *Runx3*$^{-/-}$ gastric epithelial cells in detail and found that some of them differentiated into intestinal type cells while *Runx3*$^{+/+}$ cells did not. These results suggest that IM is formed by transdifferentiation of gastric epithelial cells which express *Runx3* at very low level. Considering that *Runx3*$^{-/-}$ gastric epithelial cells are at preneoplastic condition, IM may not be a paraneoplastic lesion, but rather it may be formed as a result of preneoplastic change of gastric epithelial cells.

Materials and methods

Establishment of gastric epithelial cell lines. *Runx3*- and *p53*-deficient mice with C57BL/6 genetic background were generated as described previously [11,14]. All animal experiments were carried out in accordance with the guidelines for the care and use of laboratory animals of University of Tokyo. *Runx3*$^{+/-}$*p53*$^{+/-}$ mice were mated to obtain *Runx3*$^{-/-}$*p53*$^{-/-}$ and *Runx3*$^{+/+}$*p53*$^{-/-}$s fetuses. Gastric epithelial tissues were separated from attaching mesenchymes by treating the gastric tissue fragments with 30 mM EDTA–Hanks' solution [15]. Gastric epithelial cells from a 16.5-day fetus were separately cultured in wells precoated with rat tail collagen gels as described previously [16] with a slight modification. Briefly, cells were seeded in Ham's F12 medium (Sigma) supplemented with 10% horse serum (Trace Biosci., Australia), bovine pituitary extract (100 μg/ml; Gibco-BRL), epidermal growth factor (10 ng/ml; Upstate Biotech.), insulin (3 μg/ml; Sigma), cholera toxin (300 ng/ml; List Biol. Lab.), and hydrocortisone (3 μg/ml; Sigma), and cultured in a humidified atmosphere of 5% CO_2 in air at 37 °C. Rapidly growing cells were subcultured by treating them with trypsin–EDTA, and re-seeding dispersed cells on new collagen gels in the first 5–7 passages, then on plastic substratum after 6–8 passages. It should be noted that a cell line was established from whole stomach epithelial tissue of a mouse fetus, that cells were quite heterogeneous in nature, and that the cells seemed more homogeneous in appearance with the increase in passage number. When the cells began to proliferate rapidly on plastic substratum, usually at about passage number = 12, the culture medium was changed to Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Sigma), since the cells grew faster in the medium. Clonal cell lines were obtained from GIF-5 *Runx3*$^{-/-}$*p53*$^{-/-}$ cells by limiting dilution method.

Differentiation of the cell lines in vitro and in nude mice. The differentiation and morphogenesis of the cell lines in vitro was examined by culturing cells between collagen gels, since we found that morphogenetic potential of a colon cancer cell line could be observed with this method [17]. Briefly, collagen gel was formed on a membrane filter (Millipore, HAWP) and cloning rings were placed on it. Cells were seeded in the ring at 10^4 cells per ring to attach to the substratum (day 0). On day 1, after cloning rings were removed and culture medium was sucked off, cells were overlaid with a new collagen solution to put the cells between two collagen gel layers. The cells and collagen gel layers on the membrane filter were laid on a stainless steel grid, and cultured for more 2–5 days at the air–liquid interface without medium change. The differentiation of the cells was also examined in vivo by subcutaneously injecting about 10^7 cells into nude mice. We have previously reported that gastric epithelial cell lines established from *Runx3*$^{-/-}$*p53*$^{-/-}$ mice were tumorigenic when injected into nude mice whereas those from *Runx3*$^{+/+}$*p53*$^{-/-}$ mice were not [11]. Thus, only differentiation of *Runx3*$^{-/-}$ cells could be examined in vivo

Histological procedures. Cells cultured between collagen gels were fixed 3–6 days after incubation, and tumors were recovered from nude mice 4–8 weeks after injection. They were fixed with 4% paraformaldehyde in PBS, and processed for histological examinations by embedding tissues in paraffin, sectioning them at 5 μm, and staining tissue sections with periodic acid Schiff (PAS)–hematoxylin, alcian blue (AB)–neutral red, and high iron diamine (HID)–AB. For immunohistochemical detection of Cdx2, some sections were treated with anti-Cdx2 antibody (BioGenex), followed by Envision+ Kit (DAKO), and counter-stained with hematoxylin. For ultrastructural studies, some tissues were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer, postfixed with 1% osmium tetroxide in the buffer, dehydrated, and embedded in Epon 812 (Taab Lab). Ultrathin sections were cut, stained with uranyl acetate and lead citrate, and observed with a JOEL 1000 electron microscope.

Results

Establishment of Runx3$^{-/-}$ *gastric epithelial cell lines*

To explore whether loss of *Runx3* affects differentiation of gastric epithelial cells, we first examined the differentiation of gastric epithelial cells in *Runx3*$^{-/-}$ mice, but could not find any significant differences in their differentiation between *Runx3*$^{-/-}$ and wild-type mice at pre- and peri-natal stages (data not shown). It was impossible to examine their differentiation in postnatal animals because *Runx3*$^{-/-}$ mice died soon after birth by unknown mechanisms [11]. We thus established gastric epithelial cell lines from *Runx3*$^{-/-}$*p53*$^{-/-}$ and *Runx3*$^{+/+}$*p53*$^{-/-}$ mice, and examined whether there was a genotype-dependent difference in their differentiation potencies. Phase contrast microscopy showed that both types of cells were relatively homogeneous in appearance, and exhibited polygonal morphology, a characteristic of epithelial cells (Figs. 1A and B). At present, their passage numbers are above 40, indicating that permanent cell lines have been established. We have so far established four *Runx3*$^{-/-}$*p53*$^{-/-}$ and three *Runx3*$^{+/+}$*p53*$^{-/-}$ cell lines.

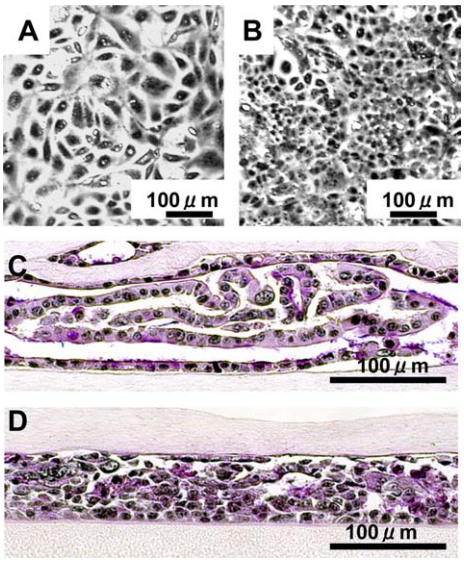

Fig. 1. Mouse gastric epithelial cell lines in vitro. (A,B) Phase contrast micrographs of: (A) a *Runx3*$^{+/+}$*p53*$^{-/-}$ cell line (GIF-9) and (B) a *Runx3*$^{-/-}$*p53*$^{-/-}$ cell line (GIF-5) in culture. (C,D) Light micrographs of: (C) GIF-9 *Runx3*$^{+/+}$*p53*$^{-/-}$ cells and (D) GIF-5 *Runx3*$^{-/-}$*p53*$^{-/-}$ cells cultured between collagen gels for 6 days, stained with PAS–hematoxylin.

Differentiation of gastric epithelial cells in vitro

When cultured between collagen gels, *Runx3*$^{+/+}$ *p53*$^{-/-}$ gastric epithelial cells formed simple columnar epithelia with occasional glandular structures. The cells exhibited polarity with PAS-positive mucus localized on the luminal surface, a characteristic of mucous neck cells, indicating that they retain the phenotype of relatively undifferentiated gastric epithelial cells. Differentiation of goblet cells was never observed (Fig. 1C). In contrast, *Runx3*$^{-/-}$*p53*$^{-/-}$ cells attached weakly to each other and did not form any glandular structures, but piled up between collagen gels. Some cells were weakly stained with PAS, indicating that they synthesized and secreted mucus, but mucus droplets were evenly distributed in their cytoplasm, suggesting that cellular polarity could not be established when cells were combined with collagen gels (Fig. 1D).

Runx3$^{+/+}$*p53*$^{-/-}$ and *Runx3*$^{-/-}$*p53*$^{-/-}$ cells responded quite differently to collagen gels, but similar responses were found in cell lines with the same genotype, indicating that Runx3 is involved in their response to collagen gels. These results also suggest that *Runx3*$^{+/+}$*p53*$^{-/-}$ cells are very similar to, but *Runx3*$^{-/-}$*p53*$^{-/-}$ cells are far different from, normal gastric epithelial cells concerning their differentiation potencies in vitro.

Differentiation of Runx3$^{-/-}$*p53*$^{-/-}$ *gastric epithelial cells in nude mice*

When subcutaneously injected into nude mice, *Runx3*$^{-/-}$*p53*$^{-/-}$ gastric epithelial cells formed tumors while *Runx3*$^{+/+}$*p53*$^{-/-}$ cells did not, a result consistent with a previous one [11]. Thus, differentiation of *Runx3*$^{-/-}$*p53*$^{-/-}$ gastric epithelial cells could be examined in vivo in nude mice.

The differentiation of *Runx3*$^{-/-}$*p53*$^{-/-}$ gastric epithelial cells differed greatly depending on the cell line, and also on the passage number in some cells. Thus in the present report, results are presented on the cells at passage number of 35–40, unless otherwise indicated. GIF-3 cells formed poorly differentiated tumors without glandular structures (Fig. 2A), while GIF-5, GIF-11, and GIF-14 cells formed well-differentiated tumors (Fig. 2G), mixed-type tumors consisted of poorly differentiated and well-differentiated cells (Fig. 2B), and well-differentiated tumors consisted of undifferentiated gastric epithelial cells (Fig. 2C), respectively.

It was remarkable that differentiation of intestinal type cells was found in tumors formed by GIF-5 (Fig. 2H) and GIF-11 cells (Figs. 2D–F). Intestinal type cells could be easily distinguished from gastric epithelial cells by the presence of goblet cells with large PAS-positive, AB-positive, and HID-positive mucus droplets while gastric type cells were characterized by PAS-positive, AB-negative, and HID-negative apical granules (Figs. 2D–F). Tumorigenicity of the cells could be reproducibly found independent of their passage number, but differentiation of intestinal type cells was found only in tumors formed by the cells with passage number >18, and was not detected in those formed by the cells with passage number <15. Differentiation of intestinal type cells was first found in tumors at 6 weeks after injection and was prominent at 8 weeks. Intestinal type cells were always located in the center of a tumor, while gastric type cells at the periphery (Figs. 2G and H), indicating that differentiation of intestinal type cells was induced by environmental factors located in the center of tumors, such as lack of nutrients and/or oxygen.

Immunohistochemical analysis showed that *Cdx2* was expressed by intestinal type cells in the center of tumors formed by GIF-5 and GIF-11 cells (Fig. 2I). We suppose that differentiation of gastric epithelial cells became less stable during propagation in vitro in the absence of *Runx3*, and that some cells in the center of a tumor responded to local factor(s) to express *Cdx2*, which induced the differentiation of intestinal type cells.

Establishment of clonal gastric epithelial cell lines and their differentiation in nude mice

As stated earlier, *Runx3*$^{-/-}$*p53*$^{-/-}$ gastric epithelial cells were heterogeneous in nature. Thus, it is possible that undifferentiated intestinal cells were contaminated in gastric epithelial cells in culture, and that such cells differentiated into intestinal type cells in response to environmental factors. To confirm that intestinal type cells were differentiated from gastric epithelial cells, clonal

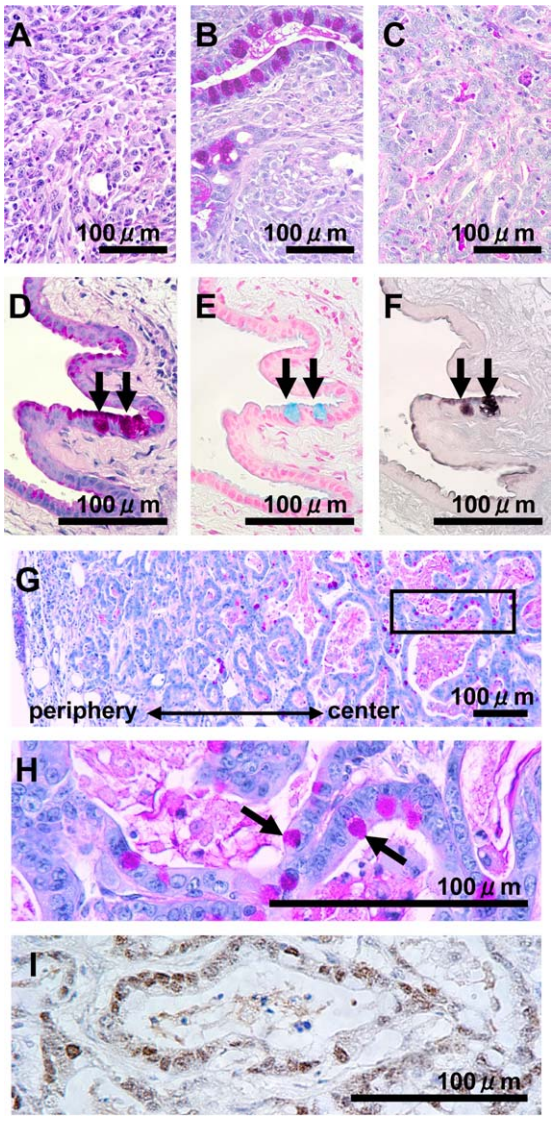

Fig. 2. Light micrographs of tumors formed by: (A) GIF-3, (B, D–F) GIF-11, (C) GIF-14, and (G–I) GIF-5 *Runx3*$^{-/-}$*p53*$^{-/-}$ cells in nude mice at 8 weeks after injection. Tissue sections are stained with (A–D, G, and H) PAS–hematoxylin, (E) AB–neutral red, (F) HID–AB. or (I) anti-Cdx2 antibody. (D–F) are neighboring three sections of a tumor formed by GIF-11 cells at passage 18, showing goblet cells (arrows) with large PAS-positive, AB-positive, and HID-positive mucus droplets among gastric type cells with PAS-positive, AB-negative, and HID-negative mucus. (H) represents a higher magnification of a part of (G), indicated by a rectangle. Goblet cells (arrows in H) are found only in the center of a tumor.

cell lines were established from GIF-5 *Runx3*$^{-/-}$*p53*$^{-/-}$ gastric epithelial cells, and their differentiation in nude mice was examined. We found that most clonal cell lines differentiated into gastric type cells with PAS-positive AB-negative mucus, but that some (about 30%) exhibited intestinal phenotypes since they produced AB-positive mucus in nude mice. Differentiation of goblet cells with full ultrastructural features including accumulation of mucous granules at the top and a compressed nucleus

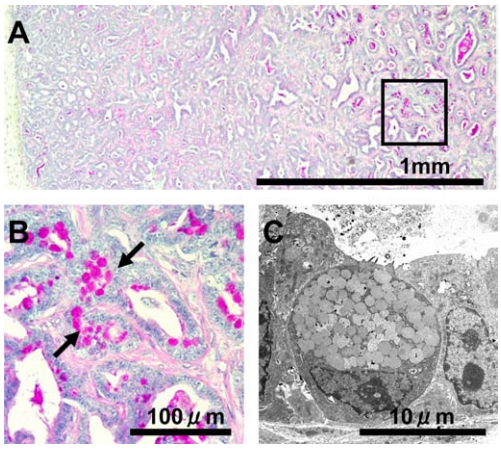

Fig. 3. Differentiation of a clonal cell line (K-3) established from GIF-5 *Runx3*$^{-/-}$*p53*$^{-/-}$ cells in a nude mouse at 8 weeks after injection. (A,B) Tissue sections are stained with PAS–hematoxylin. (B) Higher magnification of a part of (A), indicated by a square. Goblet cells (arrows in B, TEM in C) are found only in the center of a tumor.

Table 1
Characteristics of tumors formed by clonal cell lines isolated from GIF-5 *Runx3*$^{-/-}$*p53*$^{-/-}$ cells

Glandular structure formation	93% (13/14)[a]
Secretion of PAS-positive mucus	79% (11/14)
Secretion of AB-positive mucus	36% (5/14)
Differentiation of goblet cells	21% (3/14)

[a] We obtained 17 clonal cell lines from the cells. When subcutaneously injected into nude mice, 14 out of 17 lines formed tumors at 8 weeks after injection.

at the base of a cell (Fig. 3C) was found in a few (about 20%) cell lines (Table 1). In such cases, intestinal type cells were first found at 6 weeks after injection and were prominent at 8 weeks. They were located in the center of a tumor and gastric type cells at the periphery (Figs. 3A and B). These results demonstrate that intestinal type cells were transdifferentiated from *Runx3*$^{-/-}$ gastric epithelial cells. The conclusion that gastric and intestinal type cells were formed from the same germ cell is consistent with the idea of Tatematsu et al. [7], and suggests that differentiation pathway of *Runx3*$^{-/-}$ gastric epithelial cells is fragilely controlled and easily altered by environmental factors. It is interesting that critical function of Runx proteins in lineage specification and homeostasis has been shown in developing lymphocytes [18]. Thus, Runx proteins may play an essential role in the lineage specification of gastro-intestinal epithelial cells.

Discussion

In the present study, we established *Runx3*$^{-/-}$*p53*$^{-/-}$ and *Runx3*$^{+/+}$*p53*$^{-/-}$ gastric epithelial cell lines from fetal mice, and found that they were quite different in

their morphogenetic potencies when cultured with collagen gels. $p53^{-/-}$ epithelial cell lines have been established from various organs including colon [19], but establishment of gastric epithelial cell lines has not been reported. $Runx3^{+/+}p53^{-/-}$ cells formed simple columnar epithelia in combination with collagen gels, suggesting that they retain characteristics very similar to those of normal gastric epithelial cells. Thus, they would be useful for the future study on the mechanism of differentiation of gastric epithelial cells.

We also found in the present investigation that $Runx3^{-/-}p53^{-/-}$ gastric epithelial cells attached weakly to each other and could not form glandular structures in combination with collagen gels in vitro, indicating that intercellular communication is impaired in the $Runx3^{-/-}p53^{-/-}$ gastric epithelial cells. Thus, Runx3 may play a role in cell-to-cell communication, a notion which has not been presented. We have previously shown that loss of *RUNX3* is an important step in the genesis and progression of human gastric cancer [11]. It is well known that the impairment of cell-to-cell communication is an important step in the initiation and progression of cancer [20,21]. Then, impairment of cell-to-cell communication induced by the loss of *RUNX3* may contribute to the genesis and progression of human gastric cancer.

In contrast to the observation in vitro, some $Runx3^{-/-}p53^{-/-}$ gastric epithelial cell lines formed glandular structures when subcutaneously injected into nude mice, indicating that they can exhibit morphogenetic potencies in combination with skin fibroblasts in nude mice. The importance of epithelial–mesenchymal interaction in the differentiation and morphogenesis of gastro-intestinal epithelium has been well demonstrated [22]. That $Runx3^{-/-}p53^{-/-}$ gastric epithelial cells could form glandular structures only when they were combined with skin fibroblasts suggests that factors provided by fibroblastic cells are crucial for their morphogenesis, and possibly for their differentiation. The identification of the factor remains to be explored.

In the present study, we found that some $Runx3^{-/-}p53^{-/-}$ gastric epithelial cells differentiated into intestinal type cells in nude mice. Since $Runx3^{+/+}p53^{-/-}$ cells were not tumorigenic, we could not examine their differentiation in nude mice. Then at present we cannot conclude that lack of *Runx3* is the cause of differentiation of intestinal type cells from gastric epithelial cells, because we cannot demonstrate that $Runx3^{+/+}p53^{-/-}$ gastric epithelial cells never differentiate into intestinal type cells when cultured for more than 6 weeks with fibroblastic cells. It is very difficult to maintain non-tumorigenic gastric epithelial cells for a long time not only in vitro but also in vivo, since full differentiation of the cells could be observed 1 week after transplantation [23], and most cells ceased to proliferate by 2–3 weeks (unpublished observation). To address the issue, we have to establish a culture system where $Runx3^{-/-}$ gastric epithelial cells differentiate into intestinal type cells, and to examine whether expression of *Runx3* affects the differentiation. Attempts are now in progress to establish the culture system. Using the system, we will directly demonstrate that loss of *Runx3* is the cause of differentiation of intestinal type cells from gastric epithelial cells.

It is well known that the differentiation of mouse gastric epithelial cells is very stable and IM cannot be easily induced in the mouse stomach. We have previously reported that the developmental fate of gastric epithelia cannot be affected by heterologous mesenchymes in fetal mice [24]. Yamamoto et al. [25] have shown that IM has never been found in carcinogen-treated mouse stomachs, even when gastric tumors could be induced in them. We also found in the present study that differentiation of goblet cells was never found in $Runx3^{+/+}p53^{-/-}$ gastric epithelial cells in culture (Fig. 1C). There are so many gastric epithelial cell lines, but none has been reported to differentiate into intestinal type cells, so far as we know. Then, it is not probable that $Runx3^{+/+}p53^{-/-}$ gastric epithelial cells differentiate into intestinal type cells in any culture conditions, and it is remarkable that $Runx3^{-/-}p53^{-/-}$ mouse gastric epithelial cells differentiated into intestinal type cells, though it took weeks for the differentiation and many factors might be involved in the process. It is not probable that loss of *p53* affects the differentiation of gastric epithelial cells, because differentiation of gastric epithelial cells is normally controlled in $p53^{-/-}$ mice [26] and no IM was found in $p53^{-/-}$ mouse stomachs even when preneoplastic changes and adenomas were induced in the stomach by carcinogens [27].

We found in the present study that *Cdx2* was expressed in the differentiated intestinal type cells (Fig. 2I). Thus, loss of function of Runx3 may be the key event in inducing expression of *Cdx2* in the stomach, which results in the differentiation of intestinal type cells. In the normal gastric mucosa, expression of *Cdx2* may be directly or indirectly suppressed by Runx3.

We have previously reported that *RUNX3* expression is greatly reduced in IM in human gastric tissues, compared with normal epithelial cells [11]. In the present study, we found that $Runx3^{-/-}$ gastric epithelial cells differentiated into intestinal type cells. These results suggest that Runx3 is deeply involved not only in gastric carcinogenesis but also in the formation of IM in both humans and mice. We propose that gastric cancers and IMs may be derived from common gastric epithelial cell population which expresses *Runx3* at very low level (Fig. 4). We have previously shown that $Runx3^{-/-}$ gastric epithelial cells are at preneoplastic condition, because $Runx3^{-/-}p53^{-/-}$ gastric epithelial cells formed tumors in nude mice, whereas $Runx3^{+/+}p53^{-/-}$ cells did not [11]. Then, IM may not be a paraneoplastic lesion,

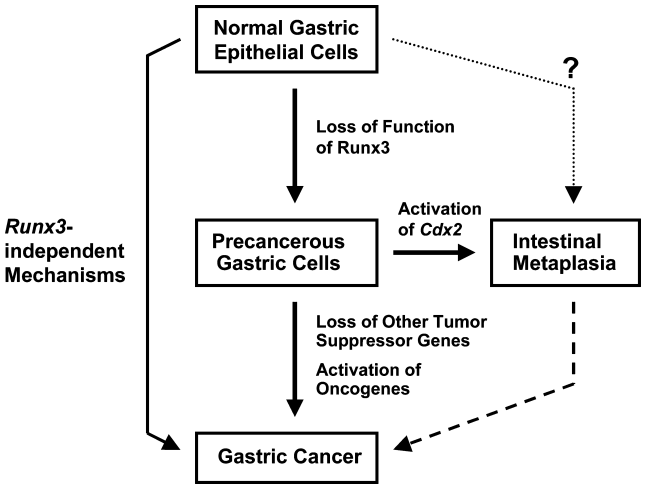

Fig. 4. Our model on the relationship between loss of function of Runx3, formation of intestinal metaplasia, and gastric cancer. Gastric cancer and intestinal metaplasia are derived from common gastric epithelial cell population which expresses *Runx3* at very low level.

but rather it may be formed as a result of preneoplastic change of gastric epithelial cell populations. This indicates that detection of IM would be useful to judge whether the gastric epithelial cells are at preneoplastic condition or not.

We have reported that expression of *RUNX3* in human gastric carcinomas was greatly reduced in about 40% of cases at stage I [11]. Thus, neoplastic gastric epithelial cells could also be induced by other mechanisms independent of Runx3, where preneoplastic cells may not form IMs. Further studies are needed to explore the mechanism of gastric carcinogenesis in detail, but this system with *Runx3*$^{-/-}$ gastric epithelial cells would be useful for the future study.

Acknowledgments

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