Review Article

Stem cells and gastric cancer: Role of gastric and intestinal mixed intestinal metaplasia

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All of the different types of stomach epithelial cells are known to be derived from a single progenitor cell in each gland. Similarly, cancers develop from single cells, based on data from clonality analysis in C3H/HeN↔BALB/c chimeric mice. Using gastric and intestinal epithelial cell markers, intestinal metaplasia (IM) can be divided into two major types: a gastric and intestinal (GI) mixed type, and a solely intestinal (I) type. Ectopic expression of *Cdx* genes and down-regulation of *Sox2* in isolated single GI mixed IM glands suggests abnormal differentiation of stem cells that can produce both gastric (G) and I type cells. Similarly, phenotypic expression of gastric cancer cells of each histological type can be clearly classified into G and I type epithelial cells. The heterogeneity of phenotypic expression of gastric cancer cells in individual cancers is assumed to reflect this intrinsic potential for differentiation in two directions. Gastric cancers at early stages, independent of the histological type, mainly consist of G type cells, and phenotypic shift from G to I type expression is clearly observed with progression. The data thus suggest IM may not be a preneoplastic change in gastric carcinoma, but rather that cells of the I type may appear independently in the gastric mucosa in IM and in gastric cancers. Intestinalization of gastric mucosa and cancer cells may represent a kind of homeotic transformation. Whether disturbance of the regulation of *Sox2* and *Cdx* genes may be of importance to the biological behavior of gastric cancers should therefore be clarified in future studies. (Cancer Sci 2003; 94: 135–141)

Based on assessment of the differentiation status of tumor cells, they appear to deviate little from their normal progenitors and to show similar differentiation programs. Studies on tissues undergoing continuous cell renewal suggest that cancer cells may originate from a stem cell compartment. Human gastric carcinomas have been classified by Lauren[1] into two major groups, the "intestinal" and "diffuse" types. It has been suggested that "intestinal" type carcinomas arise in intestinalized mucosa, whereas the "diffuse" type ones develop from the gastric mucosa proper.[1] However, recent studies on clonality of gastric cancers[2] and phenotypic expression of each intestinal metaplastic or stomach cancer cell have pointed to several contradictions.[3–6] It is therefore important to determine to what extent neoplasia relies on the presence of a stem cell compartment in epithelial tissues in terms of organ carcinogenesis. Examination of phenotypic expression of intestinal metaplasia (IM) and stomach cancers might in theory reveal the histogenesis of gastric cancers in this light.

Gastric and intestinal epithelial cell markers

Paradoxical concanavalin A staining opened up new fields in the study of phenotypic expression of epithelial cells in the alimentary tract.[7] Using this method, mucins in the alimentary tract can be divided into two main classes: class III mucins in mucous neck cells, pyloric gland cells and Brunner's gland cells and class II mucins in surface mucous cells, goblet cells, and the surface coat of intestinal absorptive cells. With other more recent developments in mucin histochemistry and immunohistochemistry, intestinal metaplasic cells and stomach cancer cells can now be clearly classified into a gastric epithelial cell type (G type), resembling pyloric gland cells and surface mucous cells, and an intestinal epithelial cell type (I type), like goblet and intestinal absorptive cells, by the analysis of phenotypic expression (Table 1 and Fig. 1). Fig. 1A shows representative histology for gastric mucosa, consisting of the foveolar cells in the upper two-thirds and pyloric gland cells in the lower one-third. Concerning gastric phenotypic markers, the surface mucous cell type contains galactose oxidase-Schiff (GOS) and sialidase-GOS reactive mucin, being positive for MUC5AC (Fig. 1B). Cells of pyloric gland cell type contain class III mucin, being positive for MUC6 (Fig. 1D), and show pepsinogen reactivity. Fig. 1E exhibits intestinal metaplastic mucosa consisting of absorptive cells with brush border, goblet cells with clear round cytoplasm containing mucin, and sometimes Paneth cells (not shown) harboring red granules in their cytoplasm that usually appear at the bottom of the glands. Regarding intestinal markers, the goblet cell type contains mucin that is GOS-negative and sialidase-GOS reactive, possessing sialyl-Tn antigen and MUC2 core protein (Fig. 1G). Cells of intestinal absorptive cell type demonstrate sucrase and intestinal type alkaline phosphatase activity, harboring CD10 as a surface marker and the structural protein villin (Fig. 1H). Cells of Paneth cell type are reactive with anti-defensin antibodies.[8–15]

Stem cells of gastric mucosa and clonal growth of gastric cancer

Gastric cancer cells show heterogeneity in terms of gastric and intestinal phenotypic markers as mentioned above. However, it is not clear whether they consist of different types of cells or are derived from a multipotential cell. To investigate the cellular origin of tumors, mosaicism of cellular genetic markers is often used. One cellular phenotypic mosaicism in females is caused by random inactivation of one X-chromosome.[16–18] Another approach is to use chimeric animals, produced experimentally by the amalgamation of cells from allelically different strains. Recently, numerous histological markers have been used for analysis of mosaicism in chimeric mice. Antibodies strictly recognizing C3H strain-specific antigens (CSAs) established by Kusakabe *et al.*[19] enable immunohistochemical discrimination of C3H cells in histological sections of chimera mouse tissues. In normal gastric and intestinal[20] mucosa of chimera mice, each gland is composed entirely of CSA-positive

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Table 1. Markers for gastrointestinal epithelial cells

Tissue types	Cell types	Markers
Gastric	Foveolar	MUC5AC, GOS[1)]
	Pyloric	MUC6, PCS[2)]
Intestinal	Absorptive	Sucrase, I-ALP[3)], CD10, villin
	Goblet	MUC2, sialidase-GOS, sialyl-Tn antigen
	Paneth	Defensin

1) GOS, galactose oxidase-Schiff.
2) Paradoxical concanavalin A staining (class III mucin).
3) I-ALP, intestinal type alkaline phosphatase.

or negative cells and no mixed glands are found, indicating that each individual gland in the adult mouse is derived from a single progenitor cell (Fig. 2A). Surface mucous cells (foveolar epithelial cells), mucous neck cells, parietal cells and chief cells in the fundic glands thus all arise from the same cell. Similarly, surface mucous cells and pyloric gland cells in each pyloric gland are from a single progenitor cell. Cancers in C3H/HeN↔BALB/c chimeric mice treated with *N*-methyl-*N*-nitrosourea (MNU) were also found to be composed of only one parental type (Fig. 2B).[2)]

Gastric and intestinal mixed type intestinal metaplasia

IM has been extensively studied as a possible premalignant condition in the human stomach.[21–24)] However, many questions remain regarding the pathogenesis of IM development as well as its relationship to gastric cancers. It is in fact heterogeneous in nature and can present as miscellaneous forms, with various authors proposing different definitions and criteria for classification. Most have focused on the similarities of IM to small or large intestinal epithelial cells morphologically, histochemically, or enzymatically.

The present widely applied classification, into complete and incomplete types, was first proposed by Kawachi and colleagues.[25, 26)] Classification based upon mucin secretion patterns as well as morphology has also allowed division into a small intestine type and a colonic type.[27, 28)] Jass and Filipe[29)] described three grades of IM (type I, II, and III) on the basis of morphology and classical mucin staining using the periodic acid-Schiff, Alcian blue, and high iron diamine methods. Type I corresponds to the complete and types II and III to the incomplete type, respectively, with mild and severe distortion of the glandular structures. While these classifications are generally accepted, they are only based upon the intestinal properties and do not take into account the gastric properties that are still preserved in association.

We have therefore proposed a new classification based upon the cell differentiation status using both gastric and intestinal cell phenotypic markers.[13, 14)] With this classification, IM is divided into two major types; a gastric and intestinal (GI) mixed type, and a solely intestinal (I) type. To confirm this histological classification, stomach mucosa was subjected to a gland isolation technique to show the successive character of the gland consisting of gastric and intestinal phenotypic cells from the top to the bottom. The isolated glands were classified into gastric (G), GI mixed, and I types according to the preservation of

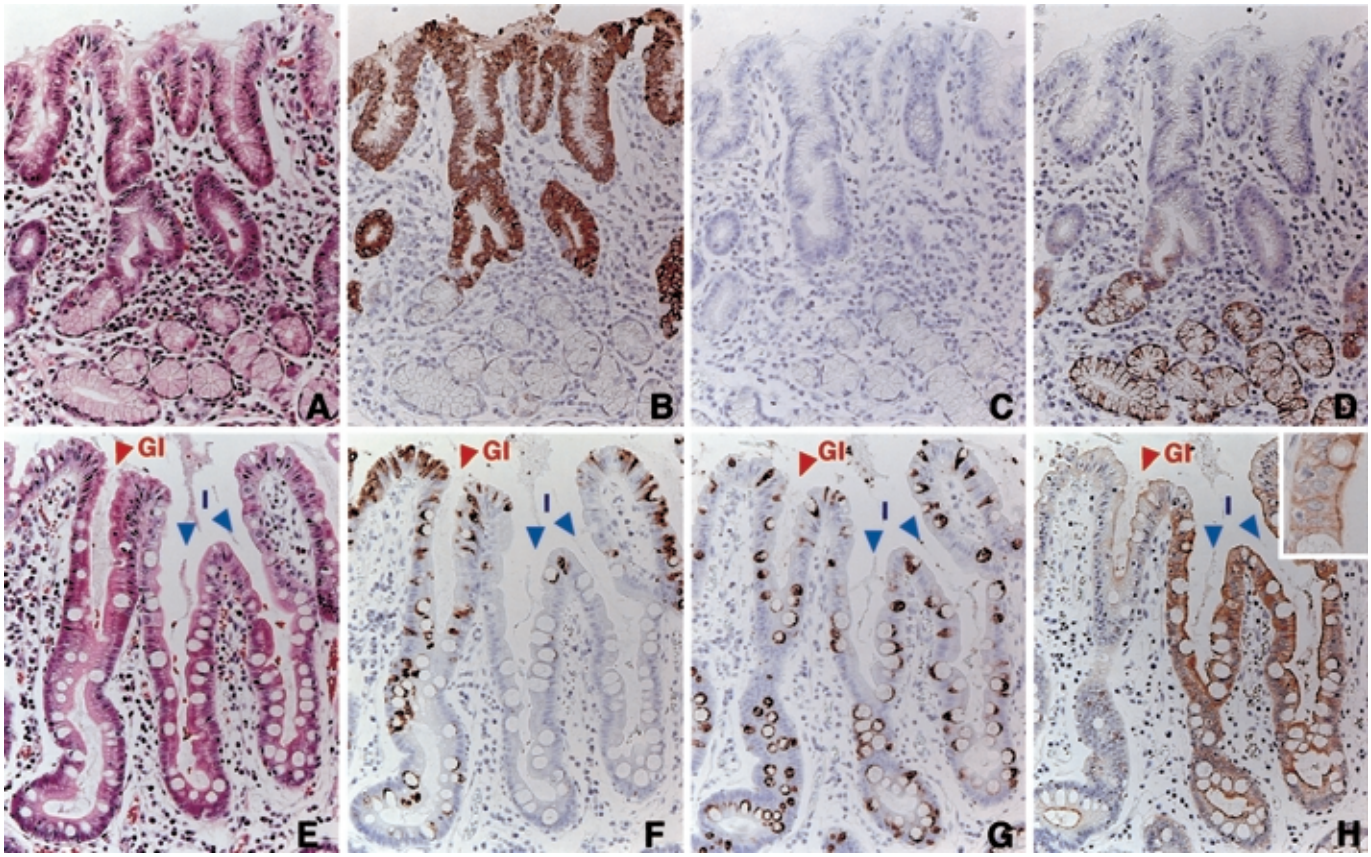

Fig. 1. Immunohistochemical staining of normal human gastric mucosa and intestinal metaplasia. (A–D) Normal pyloric mucosa showing gastric phenotype. (E–H) GI mixed type and I type intestinal metaplastic glands. H&E staining (A and E). Immunohistochemical analysis using antibodies against MUC5AC (B and F) and MUC6 (D) for gastric markers and those against MUC2 (C and G) and villin (H) to show intestinal phenotype. Visualized with 3,3′-diaminobenzidine (DAB) and counterstained with hematoxylin. GI (red arrowhead), GI mixed type IM; I (blue arrowheads), I type IM. Original magnification, ×200 (A–D) and ×160 (E–H). See Table 1.

pyloric glands and appearance of goblet cells as revealed with Alcian blue and paradoxical concanavalin A staining (Fig. 3, A–C, respectively). The G type preserves the pyloric glands without emergence of goblet cells (Fig. 3A). In the I type, intestinal metaplastic glands consisted of goblet and intestinal absorptive cells with or without Paneth cells (Fig. 3C). In the GI mixed type, on the other hand, gastric phenotype cells are found sharing the same metaplastic glands with intestinal phenotype cells in various combinations (Fig. 3B). All of the subtypes of GI mixed type IM and a subtype of the I type without Paneth cells belong to the incomplete IM category while the subtype of I type with Paneth cells corresponds to complete type IM. In many cases of the GI mixed type, atrophied pyloric glands are present under the intestinalized cells.

A mixture of gastric and intestinal phenotypes occurs at the cellular level as well as at the glandular level, involving cells of gastric phenotype together with those of intestinal phenotype as mentioned above in GI mixed type IM. Intestinal metaplastic glands are easily found with hematoxylin and eosin (H&E) staining by the presence of goblet cells and brush border lining the apical side of the epithelium (Fig. 1E). Goblet cells are confirmed to exhibit intestinal phenotype, as shown with MUC2 immunostaining (Fig. 1G), which is not present in gastric epithelium (Fig. 1C). Brush border is positive for villin, as is normal intestinal epithelium (Fig. 1H). However, gastric mucin sometimes remains in both goblet and absorptive cells as revealed with MUC5AC immunohistochemistry (Fig. 1F, red arrowhead), villin expression being weaker in such a gland (Fig.

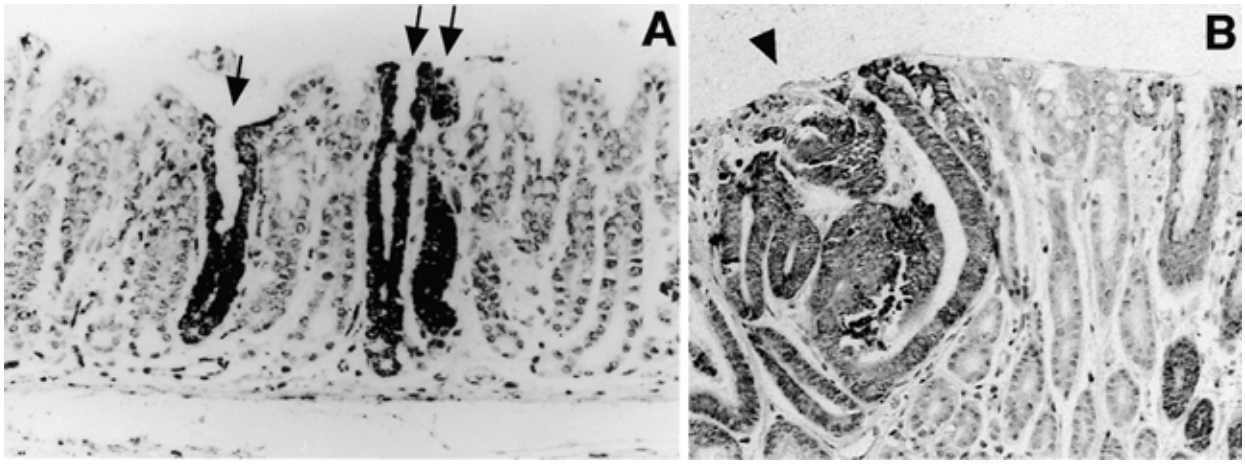

Fig. 2. Analysis of cellular origin in a C3H↔BALB/c chimera mouse with an antibody against C3H strain-specific antigen (CSA). (A) Normal pyloric mucosa. Each gland is composed entirely of CSA-positive (arrows) or -negative cells. (B) MNU-induced stomach adenocarcinoma consisting of CSA-positive cells (arrowhead) adjacent to CSA-negative glands showing monoclonal origin of tumor. Original magnification, ×200 (A) and ×100 (B).

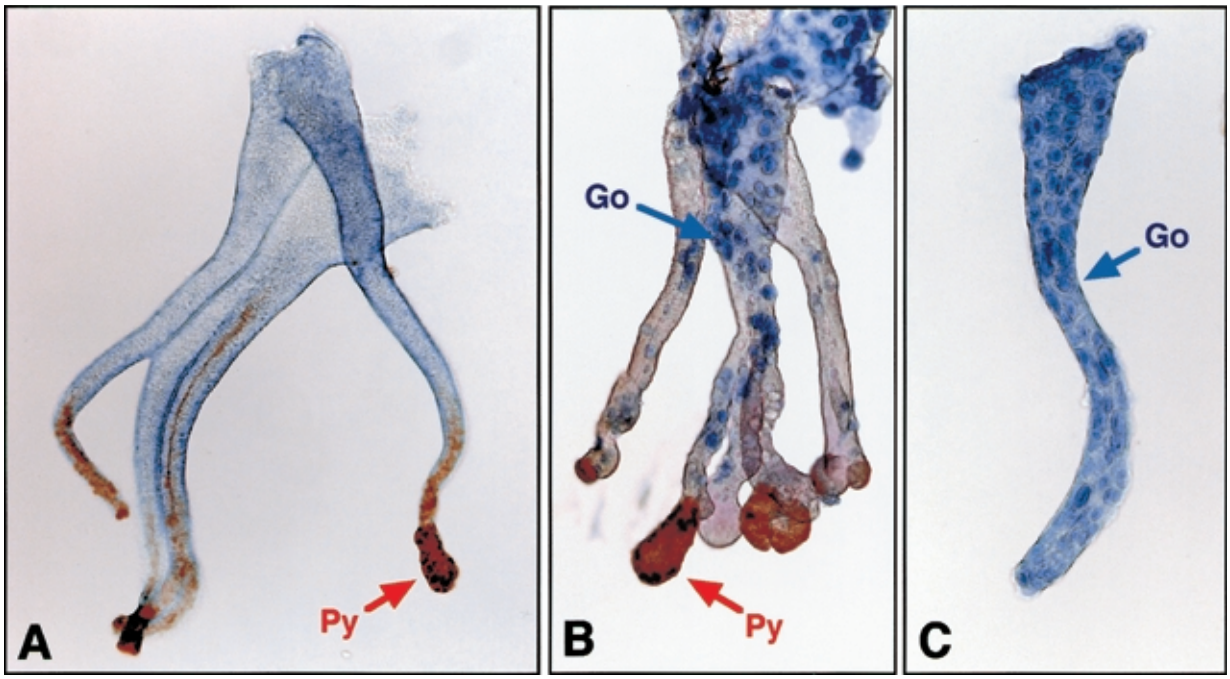

Fig. 3. Morphological and mucin histochemical classification of isolated pyloric glands from human stomach. (A) G type glands consisting of surface mucous cells in the upper part and pyloric gland cells (Py) at the bottom. (B) Glands with GI mixed IM still preserving pyloric gland cells with emergence of goblet cells (Go). (C) I type IM glands losing pyloric glands but with many goblet cells appeared. Goblet cells are shown in blue with Alcian blue staining and pyloric gland cells containing class III mucins are stained brown using DAB utilizing paradoxical concanavalin A staining.

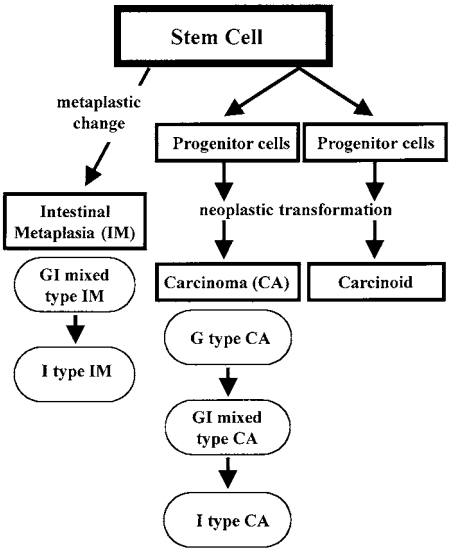

Fig. 4. Schematic illustration of the hypothesis for pathways of intestinal metaplasia and intestinalization of gastric carcinoma based on the pathological findings of human materials and experimental results with rodent models. Intestinalization progresses from GI mixed type toward I type in non-cancerous (left flow) and cancerous (middle flow) tissue independently. GI mixed type IM, gastric and intestinal mixed type intestinal metaplasia; I type IM, solely intestinal type intestinal metaplasia; G type CA, gastric epithelial cell type carcinoma; GI mixed type CA, mixed gastric and intestinal epithelial cell type carcinoma; I type CA, intestinal epithelial cell type carcinoma.

1H, red arrowhead) compared to ones without MUC5AC expression (Fig. 1, F and H, blue arrowheads). The intestinal metaplastic gland still preserving gastric mucin is also considered to be GI mixed type IM at the cellular level, whereas the others are considered to be I types, indicating the presence in the gland of a phenotypic shift from gastric toward intestinal. Thus, IM subtypes may be considered as not independent entities, but rather a sequence of pathological states with a gradual change from stomach to intestinal character. Experimentally, a phenotypic shift from GI mixed type to an I type IM could be clearly observed on sequential observation of rat stomach treated with X-rays.[6] GI mixed type IM may be composed of a mixture of cells with various degrees of intestinal phenotypic shift rather than just a random mixture of gastric and intestinal type cells. This allows us to introduce the notion that IM might be due to abnormal stem cell differentiation still obeying a certain order (Fig. 4, left flow).

It is believed that stem cells (multipotent progenitor cells) are present in the proliferative cell zone in the isthmus region of the gastric glands, giving rise to all the various cell types by differentiation, so that consequently gastric glands are monoclonal in the adult stage.[30, 31] In the environment of a normal gastric gland, cells derived from stem cells undergo complex bipolar migration from the isthmus either upward or downward. In pyloric mucosa, surface mucous cells move upward, while pyloric gland cells migrate downward.[32] In the crypts of the small intestine, on the other hand, stem cells would be expected to be present in the proliferative cell zone at the bottom of the crypts. In the normal intestinal gland, cells that will become absorptive and goblet cells move up, and only these differentiating into Paneth cells migrate lower from the proliferative cell zone. In GI mixed IM, gastric surface mucous cells, intestinal absorptive cells and goblet cells are found in the upper glandular portions from the proliferative zone and pyloric gland cells and Paneth cells are found in the lower glandular portions from the proliferative zone (Fig. 5, A and B).[14] GI mixed type IM might be the consequence of abnormal differentiation of stem cells that can produce both gastric and intestinal type cells, with the normal cell migration pattern preserved. Since epithelial cell differentiation and migration of gastric glands are thought to be closely linked, it is not clear why only the former is disturbed. Recently, epithelial-mesenchymal interactions have attracted attention due to their alteration under miscellaneous conditions, as well as interest in their role in organogenesis.[33] It clearly warrants further study whether the organization of intestinal metaplastic lesions might be influenced by interstitial cells.

Expression of homeobox genes in intestinal metaplasia

Caudal genes are necessary for determination of anteroposterior polarity during early *Drosophila* development[34] and caudal-type homeobox (*Cdx*) 1 and *Cdx2* are mammalian members

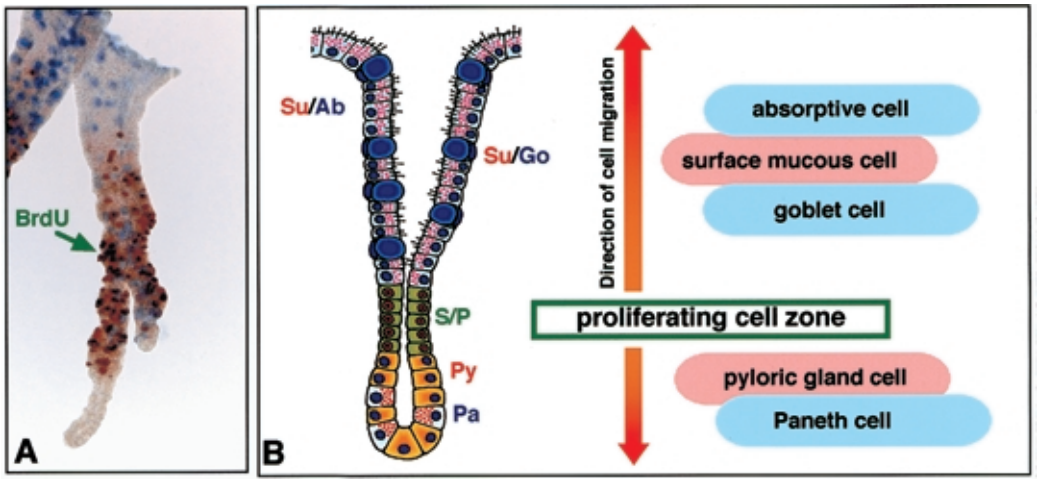

Fig. 5. Direction of cell migration and differentiation of gastric and intestinal phenotypic cells in GI mixed IM. (A) Isolated GI mixed intestinal metaplastic glands are incubated in the presence of 10 mg/ml bromodeoxyuridine (BrdU) for 2 h. Proliferating cells are visualized with BrdU immunohistochemistry. Goblet cells are visualized with Alcian blue. Original magnification, ×100. (B) Schematic view of the cell differentiation (left panel) and direction of cell migration in GI mixed intestinal metaplastic glands consisting of both gastric (highlighted red) and intestinal (highlighted blue) phenotypic cells (right panel). Su, surface mucous cells; Ab, absorptive cells; Go, goblet cells; Su/Ab, absorptive cells retaining mucin of surface mucous cells; Su/Go, goblet cells containing both gastric and intestinal mucin; S/P, stem cells and proliferating cells; Py, pyloric gland cells; Pa, Paneth cells.

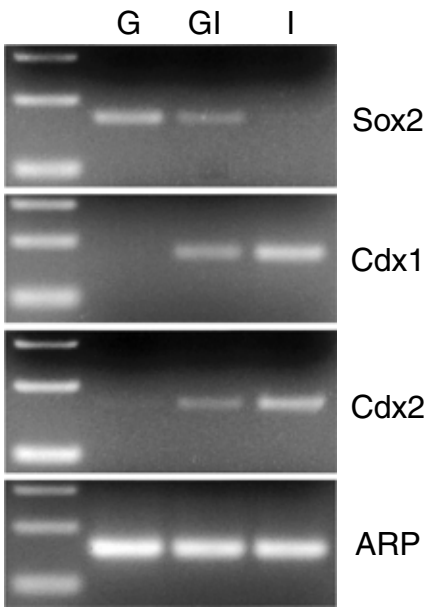

Fig. 6. Semi-quantitative reverse transcription-polymerase chain reaction of *Sox2*, *Cdx1*, and *Cdx2* transcripts in isolated stomach glands. G type (lane G), GI mixed type (lane GI), and I type glands (lane I). *Sox2* is expressed in gastric epithelium, is down-regulated when IM starts, and finally disappears in I type glands. On the other hand, *Cdx* genes are ectopically transcribed in intestinal metaplastic glands of both GI mixed and I types, being upregulated with the progression towards the I type. Acidic ribosomal phosphoprotein PO (ARP) is shown as an internal control. Left lane, DNA molecular weight markers corresponding to 100, 200, and 300 base pairs from the bottom.

of the caudal-related homeobox gene family.[35] In the adult mouse and in humans, expression is strictly confined to the gut, from the duodenum to the rectum. Silberg *et al.*[36] previously reported the presence of *Cdx1* protein in intestinal metaplastic lesions of the human stomach. Mizoshita *et al.*[37] demonstrated the expression of *Cdx1* and *Cdx2* in both the small and large intestine and in intestinal metaplastic mucosa of the human stomach. On the other hand, *in situ* analysis of the chicken *cSox2* gene, a member of the transcription factor family containing an *Sry*-like high-mobility group box, demonstrated localized expression in the embryonic endoderm. Transcripts of *cSox2* appear before commencement of morphogenesis and cytodifferentiation in the rostral gut epithelium from the pharynx to the stomach. The caudal limit of *cSox2* expression coincides with that of the region competent for proventricular differentiation and to the rostral limit of the domain of *CdxA*, a homologue of *Drosophila* caudal.[38] In the human digestive tract, *Sox2* expression is found in stomach epithelium but is very low in intestine, as observed in the chicken. However, in IM, *Sox2* transcripts begin to decrease and gradually disappear as IM progresses from GI mixed type to I type (Fig. 6). The expression patterns of *Sox2* and *Cdx1/Cdx2* are inversely related and down-regulation of *Sox2* could be an important mechanism in IM in addition to ectopic expression of *Cdx1/Cdx2*.[39]

Gastric and intestinal phenotypic expression of gastric cancer cells

It is widely believed that the phenotypic expression of tumor cells tends to be the same as that of the tissue of origin of the cells. Histologically, human gastric carcinomas have been classified into two major groups, the "intestinal" and "diffuse" types of Lauren,[1] which respectively nearly correspond to the "differentiated" and "undifferentiated" types of Nakamura *et al.*[40] and Sugano *et al.*[41] in Japan. Using several gastric and intestinal cellular differentiation markers as mentioned above, phenotypic expression of human and rat gastric cancer cells of each histologic type can be clearly classified into a gastric epithelial cell type (G type), including pyloric gland cells and surface mucous cells and an intestinal epithelial cell type (I type) including goblet cells and intestinal absorptive cells.[3–5] Based on findings for monoclonal growth of gastric cancers,[2] it is assumed that individual lesions are derived from a single cell with intrinsic potential to differentiate into G type and I type cancer cells. Although Lauren's classification[1] has been widely used, it is inadequate for studies of histogenesis of gastric cancers and phenotypic expression at the cellular level, because it confuses I type cancer cells with "diffuse" structure and G types with the "intestinal" type of Lauren.

Sugihara *et al.*[42] and Fujimori *et al.*[43] have revealed an organoid differentiation of intramucosal signet ring cell carcinomas. The upper and lower layers of the typical intramucosal laminated structure consist of carcinoma cells containing surface mucous cell and pyloric gland cell type mucins, respectively, whereas the middle layer is occupied by immature carcinoma cells. Such a structure appears to simulate cellular differentiation occurring in normal mucosa. Similar organoid differentiation, mimicking pyloric gland mucosa, with GI type IM and I type IM, is often found within differentiated adenocarcinomas.[44]

Phenotypic change from G to I type cancer cells in gastric cancers

Focusing on the histogenesis of gastric cancer, we have compared the frequency of IM in the surrounding mucosa in the two types to clarify whether "intestinal" type carcinomas arose in intestinalized mucosa and "diffuse" type carcinomas in the gastric mucosa proper. Phenotypic expression of gastric cancers originating from IM should be of the I type. Even if the phenotypic expression of I type cells of gastric cancer is unstable, the incidence of I type cells in small gastric cancers should be higher than that in large gastric cancers, although in fact it appears to be stable.[45] Sequential and quantitative analysis of the appearance of IM and I type tumor cells in gastric tumors during gastric carcinogenesis induced by a chemical carcinogen in rats clearly demonstrated the following (Fig. 4, middle flow).[3] (i) Adenomatous hyperplasia totally consisting of G type cells appears first. (ii) All adenocarcinomas mainly consist of G type cancer cells and in more than 50% of cases they are composed entirely of cells of G type. No tumors consisting only of I type cells were found. (iii) The incidence of I type tumor cells increased significantly in gastric lesions with progression from adenomatous hyperplasia through small well-differentiated adenocarcinomas to large well-differentiated adenocarcinomas. (iv) Tumor cells of G and I types may be present in the same acini in adenocarcinomas. (v) Adenocarcinomas with I type cells occasionally develop in pyloric mucosa in the absence of IM, and tumors without I type tumor cells sometimes occur in pyloric mucosa with IM. Therefore we earlier proposed that IM may not be a preneoplastic change in gastric carcinoma, but rather that cells of the I type may appear independently in the gastric mucosa in IM or in gastric cancers.

Recently, a similar phenomenon was also observed in human gastric cancers. Gastric cancers consisting of G type cancer cells in gastric mucosa with IM and the presence of gastric cancers consisting of I type cancer cells in ordinary mucosa suggest phenotypic expression of gastric cancer cells is independent of phenotypic changes in the surrounding gastric mucosa.[44, 46] Yamachika *et al.*[46] and Bamba *et al.*[47] found signet ring cell carcinomas at early stages to consist mainly of G type cancer cells, with a phenotypic shift from G type to I type expression accompanying increasing depth of invasion. In addition, Yoshikawa *et al.*[44] revealed over 40% of early stage Lauren's "intestinal" type carcinomas to consist mainly of G type

cancer cells, and again a phenotypic shift from G to I type expression was evident with progression.

Expression of homeobox genes in gastric carcinomas

Intestinalization of gastric cancer cells differs from the non-systematic deregulation of a single gene that is often seen during carcinogenesis. For intestinalization, changes in the expression of various genes determining cell structures and functions may be involved. Intestinalization of gastric cancer cells may represent a kind of homeotic transformation. In the course of human ontogenesis, homeobox genes play a key role in developmental processes. Fetal stomach, which develops from the foregut, displays areas of "intestinal type mucosa" with goblet cells and epithelial cells with striated borders in the antrum and cardia.[48] Intestinalization of gastric cancer cells may thus indicate a loss of ability to inhibit the primitive intestinal phenotype, which was present in the fetal tissue but is normally inhibited in adult cells. Initiation of carcinogenesis may lead to a less differentiated appearance or the capacity to differentiate in both gastric and intestinal directions, perhaps with deregulation of homeobox gene expression. Recent evidence suggests roles for several homeobox genes in processes leading to malignancy. Silberg *et al.*[36] and Bai *et al.*[49] have shown that *Cdx1* is sometimes expressed in adenocarcinomas of the stomach. Mizoshita *et al.*[50] suggested *Cdx1* to be linked with I type cancer cells in both "intestinal" and "diffuse" type lesions of Lauren's histological classification. Ectopic expression of Cdx may be related to I type phenotypic expression of cancer cells. As mentioned regarding GI mixed IM, investigation of *Sox2* expression could provide essential clues to understanding G type and GI mixed type cancer. Disturbance of regulation of *Sox2* and *Cdx1/2* within cancer originating from single cells with multipotential for differentiation might play important roles in determining phenotypic expression of gastric neoplastic cells and in the biological behavior of gastric cancers.

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