STOMACH

Cdx1 induced intestinal metaplasia in the transgenic mouse stomach: comparative study with Cdx2 transgenic mice

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Background and aims: Gastric intestinal metaplasia, which is mainly induced by *Helicobacter pylori* infection, is thought to be a precancerous lesion of gastric adenocarcinoma. Intestinal metaplastic mucosa expresses intestine specific homeobox genes, *Cdx1* and *Cdx2*, in the human gastric mucosa. We and others have reported that ectopic expression of *Cdx2* in the gastric epithelium generates intestinal metaplasia in the transgenic mouse model.

Methods: To clarify the differences in the roles of *Cdx1* and *Cdx2* in intestinal metaplasia, we generated transgenic mice expressing *Cdx1* in the gastric mucosa and compared *Cdx1* induced gastric mucosal morphological changes with *Cdx2* induced intestinal metaplasia.

Results: The gastric mucosa in *Cdx1* transgenic mice was completely replaced by intestinal metaplastic mucosa, consisting of all four intestinal epithelial cell types: absorptive enterocytes, goblet, enteroendocrine, and Paneth cells. Paneth cells, which were not recognised in *Cdx2* transgenic mice, were in the upper portion of the intestinal metaplastic mucosa. Pseudopyloric gland metaplasia, which was induced in *Cdx2* transgenic mice, was not recognised in *Cdx1* transgenic mice. Proliferating cell nuclear antigen (PCNA) positive cells were diffusely scattered in *Cdx1* induced intestinal metaplastic mucosa while PCNA positive cells in *Cdx2* induced intestinal metaplastic mucosa were in the base of the metaplastic mucosa. Intestinal metaplastic mucosa of *Cdx1* transgenic mouse stomach was significantly thicker than that of wild-type or *Cdx2* transgenic mouse stomach.

Conclusions: We have confirmed that *Cdx1* induced gastric intestinal metaplasia but that it differed from *Cdx2* induced intestinal metaplasia in differentiation, structure, and proliferation.

Gastric cancer remains the second most common cause of cancer deaths worldwide.¹ Human gastric carcinomas have been histologically classified into two main types: intestinal and diffuse.² Intestinal-type gastric carcinoma is frequently accompanied by widespread intestinal metaplasia that is mainly induced by *Helicobacter pylori* infection. Correa presented a hypothesis with respect to the mechanism of gastric carcinogenesis due to *H. pylori*.^{3–5} *H. pylori* infection is involved in the process of progression from normal gastric mucosa to superficial gastritis, chronic gastritis, atrophic gastritis, and finally to intestinal metaplasia. Intestinal-type gastric carcinoma is believed to arise via dysplasia from intestinal metaplasia.

Cdx1 and *Cdx2*, caudal related homeodomain transcription factors, are selectively localised in the mucosal epithelial nuclei of the small intestine and colon of the fetus and adult in both humans and mice.^{6–7} While normal gastric mucosa does not express transcription factors *Cdx1* or *Cdx2*, strong nuclear immunoreactivity for *Cdx1* was detected in human gastric intestinal metaplastic mucosa.⁸ Furthermore, we and others have reported expression of *Cdx1* and *Cdx2* in human intestinal metaplastic mucosa.^{9–13} These findings suggest that *Cdx1* and *Cdx2* may play a pivotal role in the development of intestinal metaplasia in the human stomach.

Aberrant expression of *Cdx2* in the stomach leading to the development of intestinal metaplasia in the transgenic mouse model was reported previously by us and others.^{14–15} In the normal intestine, *Cdx2* is expressed at high levels in differentiated epithelial cells in the villi while *Cdx1* is predominantly localised in undifferentiated cells of the proliferative crypt compartment.^{6–16–18} Absorptive enterocytes, and goblet and enteroendocrine cells are located in villi that express *Cdx2*, but not *Cdx1*, while Paneth cells are located in crypts that express *Cdx1*. Silberg *et al* reported that *Cdx2*

induced intestinal metaplasia generated goblet cells and expressed intestine specific genes, but not enteroendocrine cells and Paneth cells.¹⁵ In our *Cdx2* expressing transgenic mouse stomach, intestinal metaplasia contained enterocytes, and goblet and enteroendocrine cells, but no Paneth cells,¹⁴ indicating that *Cdx2* induces differentiation of selected lineages of intestinal epithelial cells. It is unclear whether expression of *Cdx1* in gastric mucosa might transdifferentiate the gastric lineage and, if so, comparing the nature of the *Cdx1* induced phenotype with that of *Cdx2* might provide insight into the differences in the roles of these two genes in inducing intestinal metaplasia.

In this study, we investigated the differences in the roles of *Cdx1* and *Cdx2* in gastric intestinal metaplasia by analysing transgenic mice that expressed *Cdx1* or *Cdx2* specifically in the gastric mucosa, using the H⁺/K⁺-ATPase β subunit gene promoter.

MATERIALS AND METHODS

Generation of *Cdx1* and *Cdx2* transgenic mice

pBS/HKATPase/ β globin contains nucleotides –1417 to +15 of the rat H⁺/K⁺-ATPase β subunit gene and exon 2, intron 2, and exon 3 of rabbit β globin gene in pBluescript II SK(+) (fig 1A). *Cdx1* cDNA or *Cdx2* cDNA was inserted into the EcoRI site of exon 3 of the rabbit β globin gene, yielding pBS/HKATPase/ β globin/*Cdx1* or pBS/HKATPase/ β globin/*Cdx2*. The HKATPase/ β globin/*Cdx1* insert in pBS/HKATP/ β globin/*Cdx1* or the HKATPase/ β globin/*Cdx2* insert in pBS/HKATP/ β globin/*Cdx2* was released, purified, and then used for

Abbreviations: ALP, alkaline phosphatase; HfD, high iron diamine; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; PBS, phosphate buffered saline; Tff3, trefoil family factor 3; HSV-1-tk, herpes simplex virus 1 thymidine kinase

Figure 1 (A) Structures of pBS/HKATPase/ β globin/Cdx1 and pBS/HKATPase/ β globin/Cdx2. pBS/HKATPase/ β globin contains nucleotides -1417 to +15 of the rat H^+/K^+ -ATPase β subunit gene and exon 2, intron 2, and exon 3 of the rabbit β globin gene in pBluescript II SK(+). Cdx1 cDNA or Cdx2 cDNA was inserted into the EcoRI site of exon 3 of the rabbit β globin gene, yielding pBS/HKATPase/ β globin/Cdx1 or pBS/HKATPase/ β globin/Cdx2. HKATPase/ β globin/Cdx1 or HKATPase/ β globin/Cdx2 was excised from the plasmid (pBS/HKATPase/ β globin/Cdx1 or pBS/HKATPase/ β globin/Cdx2) and injected into the oocytes. (B) Polymerase chain reaction (PCR) data from Cdx1 transgenic and wild-type mice. The presence of the Cdx1 transgene was determined by PCR using primers specific for Cdx1. (C) PCR data from Cdx2 transgenic and wild-type mice. The presence of the Cdx2 transgene was determined by PCR using primers specific for exon 3 of the rabbit β globin gene and the Cdx2 gene. (D) H^+/K^+ -ATPase (blue) and Cdx1 (brown) staining for the stomach specimen from Cdx1 transgenic mice. (E) At 30 days after birth, H^+/K^+ -ATPase positive parietal cells (blue) and Cdx1 positive cells (brown) were seen. Inset shows coexpression of H^+/K^+ -ATPase (blue) and Cdx1 (brown). However, several glands expressing only Cdx1 without H^+/K^+ -ATPase expression appeared in the fundic mucosa (arrow). (F) Some H^+/K^+ -ATPase positive glands (blue) remained in the zymogenic zones of transgenic mice at 60 days after birth. Cdx1 (brown) was diffusely expressed in gastric mucosa. Inset shows coexpression of Cdx1 (brown) and H^+/K^+ -ATPase (blue). (G) H^+/K^+ -ATPase positive parietal cells (blue) were extremely rare in zymogenic zones of transgenic mice at 90 days. Cdx1 (brown) was diffusely expressed in the gastric mucosa. Inset shows coexpression of Cdx1 (brown) and H^+/K^+ -ATPase (blue). (H) Parietal cells completely disappeared at 120 days. Cdx1 (brown) was diffusely expressed in the gastric mucosa.

pronuclear injection of 500 C57BL/6J oocytes for each insert. Injected eggs were transferred to pseudopregnant Swiss Webster females using standard techniques.¹⁹

Analyses of Cdx1 and Cdx2 transgenic mice

One hundred liveborn mice derived from HKATPase/ β globin/Cdx1 injected oocytes were screened for the presence of the Cdx1 transgene by polymerase chain reaction (PCR) using primers specific for Cdx1 (5'-GGC GGC GGT GGC AGC GGT AAG-3' (+428 to +450) and 5'-TGT GAG CCC CAG GTT AGC AG-3' (+561 to +580)) (fig 1B). Intron 1, whose size is 15146 bps, is in the genome between two Cdx1 primers while there is no intron in the transgene. Three transgenic founders were identified and three lines (pedigrees #7, #21, #78) were established.

Eighty liveborn mice derived from HKATPase/ β globin/Cdx2 injected oocytes were screened for the presence of the Cdx2 transgene by the PCR method using primers specific for exon 3 of the rabbit β globin gene (5'-CCT GGG CAA CGT GCT GGT-3') and the Cdx2 gene (5'-CCC CGG GTG CGT AGC CA-3') (fig 1C). Four transgenic founders were identified and three lines (pedigrees #17, #26, #53) were established.

Histopathology

Stomach tissue specimens were fixed in neutral buffered 10% formalin for 12–24 hours, washed in 70% ethanol, processed by standard methods, embedded in paraffin, sectioned at 3 μ m, and stained with haematoxylin and eosin or Alcian blue at pH 2.5. For mucous characterisation, Alcian blue (pH 2.5) with high iron diamine (HID) stain was applied.

To compare the thickness of the proliferative zone and gastric mucosa in wild-type, Cdx1, and Cdx2 transgenic mice, we made 10 sections from the stomachs of 10 wild-type, 10 Cdx1, and 10 Cdx2 transgenic mice sacrificed at the age of 50 weeks (350 days), respectively. We measured the thickness of the proliferative zone and gastric mucosa in the entirely longitudinally sectioned glands on each section.

Immunohistochemistry

Sections (3 µm thick) were cut, deparaffinised, rehydrated in phosphate buffered saline (PBS), placed in 10 mM citrate buffer (pH 6.0), and heated in an 850 W microwave for 10 minutes to recover antigenicity. Slides were preincubated with blocking buffer (Dako, Carpinteria, California, USA) for 15 minutes at room temperature. Primary antisera were diluted in PBS and incubated overnight at 4°C. Slides were then washed in PBS and incubated with Envision (Dako). After development with 3,3'-diaminobenzidine tetrahydrochloride (Wako Pure Chemical Industries, Osaka, Japan) or TrueBlue (KPL, Gaithersburg, Maryland, USA), slides were counterstained with haematoxylin and viewed under a light microscope.

Our panel of primary antisera included: antipepsinogen C (1:100; Bidesign, Saco, Maine, USA), anti-H⁺/K⁺-ATPase (1:100, developed in our laboratory), antiproliferating cell nuclear antigen (PCNA) (1:2000; Sigma, St Louis, Missouri, USA), anti-MUC5AC (1:30; Novocastra, UK), antiglicentin (1:100; Novocastra), antilysozyme (1:100; Novocastra), anti-Cdx2 (1:100; BioGenex, San Ramon, California, USA), and anti-Cdx1 (1:100; developed in our laboratory). Polyclonal rabbit antibody for Cdx1 was made by immunising a synthetic peptide corresponding to the carboxyl terminus of mouse Cdx1 (PSPVPVKEEFLP). The specificity of Cdx1 was confirmed by western blot analysis.

RNA isolation and reverse transcriptase (RT)-PCR

Total RNAs were isolated with ISOGEN according to the protocol provided by the manufacturers (Nippongene, Tokyo, Japan). First strand cDNAs were prepared from isolated RNAs using RT, according to the manufacturer's instructions (Toyobo, Tokyo, Japan). PCR amplification was performed at 94°C for two minutes, then 35 cycles of 94°C for 30 seconds, 60°C for 30 seconds, 72°C for 30 seconds, and 72°C for 10 minutes. The following oligonucleotides were used for PCR amplification:

- (1) villin: 5'-ATG ACT CCA GCT GCC TTC TCT-3' (sense) and 5'-GCT CTG GGT TAG AGC TGT AAG-3' (antisense),
- (2) sucrase-isomaltase: 5'-GGC AAG ATC CTG TTT CCT GGA-3' (sense) and 5'-CGA GCC TTA GGA ACA TAG CCA-3' (antisense),
- (3) trefoil family factor 3 (Tff3): 5'-AGA TTA CGT TGG CCT GTC TCC-3' (sense) and 5'-TCA GAT CAG CCT TGT GTT GGC-3' (antisense),
- (4) Muc2: 5'-GGA GAG CAC TTT GAG TTT GAC TGC-3' (sense) and 5'-GTC CTG GCA CTT GTT GGA ATA CAC-3' (antisense),
- (5) cryptdin 1: 5'-AAG AGA CTA AAA CTG AGG AGC AGC-3' (sense) and 5'-GGT GAT CAT CAG ACC CCA GCA TCA GT-3' (antisense).

The sequence of all PCR fragments was verified. PCR products were separated on 2% agarose gels.

Measurement of gastric acidity

Ten wild-type and 10 Cdx1 transgenic mice (120 days old) were denied access to food overnight and then anaesthetised with ether. After the abdominal wall was incised, the pylorus was ligated and the incision was sutured. Gastric fluid in the

stomach was collected one hour after pylorus ligation. The pH of the gastric fluid secreted over one hour was measured using a micro pH meter.

Serum gastrin concentrations

Ten wild-type and 10 Cdx1 transgenic mice (120 days old) were denied access to food overnight. Sera were obtained at the time of death and stored at -20°C until gastrin determination. Serum gastrin was quantified in duplicate by radioimmunoassay.

RESULTS

To investigate whether the intestine specific transcription factor Cdx1 can promote the development of intestinal metaplasia in the stomach, we generated transgenic mice with stomach specific expression of Cdx1 using the β subunit gene promoter of rat H⁺/K⁺-ATPase (fig 1A). Three transgenic founders (#7, #21, #78) were identified and three lines were established. All three lines of Cdx1 transgenic mice were fertile and indistinguishable from their wild-type littermates in behaviour and weight. All three lines of Cdx1 transgenic mice also exhibited almost the same histological changes in the stomach. We did not observe any differences between gastric mucosal changes of Cdx1 heterozygotic mice and those of Cdx1 homozygotic mice. For the following experiments, we used pedigree #21 and analysed Cdx1 transgenic heterozygotic mice. We also compared morphological changes of the gastric mucosa of Cdx1 transgenic mice with those of Cdx2 transgenic mice previously reported.¹⁴ We generated three lines of Cdx2 transgenic mice (#17, #26, #53), all of which showed the same morphological changes in the gastric fundic mucosa. We did not observe any differences between the gastric mucosal changes of Cdx2 heterozygotic mice and those of Cdx2 homozygotic mice. For the following experiments, we used pedigree #17 and analysed Cdx2 transgenic heterozygotic mice. The copy number of Cdx1 (pedigree #21) was 15 and that of Cdx2 (pedigree #17) 10.

Cdx1 transgenic mouse stomach was the same size as wild-type mouse stomach. To examine the relation between parietal cells and Cdx1 expressing cells, we stained gastric mucosal cells using antibodies for both H⁺/K⁺-ATPase and Cdx1. Expression of both Cdx1 and H⁺/K⁺-ATPase in the same cells of the gastric fundic mucosa was recognised immediately after birth (not shown). However, morphological changes were not detected and the number of parietal cells of Cdx1 transgenic mice was the same as that of wild-type mice until approximately 25 days after birth (not shown). At about 30 days after birth, several glands that expressed only Cdx1 appeared among the gastric fundic mucosa that expressed both Cdx1 and H⁺/K⁺-ATPase in the same cells (fig 1E). Those glands expressed only Cdx1 with complete loss of H⁺/K⁺-ATPase (fig 1E). After that, the number of glands that expressed Cdx1 and not H⁺/K⁺-ATPase gradually increased. Sixty days after birth, the number of parietal cells decreased and parietal cells remained only in several glands while Cdx1 was diffusely expressed in gastric epithelial cells (fig 1F). Parietal cells stained blue coexpressed Cdx1 (fig 1F, inset). Thereafter, the number of parietal cells further decreased and only a few parietal cells remained at 90 days (fig 1G). At 120 days, parietal cells completely disappeared and Cdx1 was diffusely expressed in the gastric mucosa (fig 1H).

To determine the functional consequences of parietal cell loss, we determined the pH of gastric fluid from Cdx1 transgenic mice at 120 days. Mean pH was 7.1 (SD 0.2), compared with 2.2 (0.2) in wild-type mice. Plasma gastrin level in wild-type mice averaged 210 (SD 20) pg/ml and that in transgenic mice 1345 (120) pg/ml. Differences in both pH

Figure 2 Fundic mucosa of wild-type, Cdx1, and Cdx2 transgenic mouse stomachs (haematoxylin-eosin staining). (A) The normal fundic mucosa of a wild-type mouse stomach at 120 days. (B) Parietal and chief cells were completely lost in the Cdx1 transgenic mouse stomach at 120 days. The mucosa was completely replaced by intestinal metaplasia that consisted of goblet cells and absorptive cells with brush border. (C) In addition to goblet and absorptive cells with brush border, pseudopyloric gland metaplasia was seen in the base of the intestinal metaplastic mucosa of Cdx2 transgenic mouse stomach at 120 days. (D) Magnified view of the metaplastic gland with brush border and goblet cells of Cdx1 transgenic mouse stomach at 120 days. Paneth cells with intracytoplasmic eosinophilic granules were seen in the upper portion of the intestinal metaplastic mucosa. (E) Paneth cells of Cdx1 transgenic mouse stomach at 120 days were stained by the antibody for lysozyme.

and plasma gastrin levels between Cdx1 transgenic and wild-type mice were statistically significant (Student's *t* test, $p < 0.001$). pH and plasma gastrin levels in Cdx2 transgenic mice were almost the same as those in Cdx1 transgenic mice.¹⁴

Gastric units located in the zymogenic zone (corpus) of wild-type mouse at 120 days after birth contain five epithelial lineages (foveolar, mucous neck, parietal, chief, and enteroendocrine cells) (fig 2A). The mucosal architecture of the zymogenic and mucoparietal zones was replaced completely by intestinal metaplasia in the stomachs of 120 day old Cdx1 transgenic mice (fig 2B). Metaplastic glands were composed of goblet cells and columnar intestinal-type epithelial cells (fig 2B). While pseudopyloric gland metaplasia was seen in intestinal metaplasia of Cdx2 transgenic mice (fig 2C),¹⁴ it was not seen in intestinal metaplasia of Cdx1 transgenic mice (fig 2B). The prominent brush border was observed on the surface of intestinal-type cells on high power magnification of metaplastic glands (fig 2D). Paneth cells were not expressed in Cdx2 transgenic mouse stomach¹⁴ but they were in the upper portion of Cdx1 induced intestinal metaplastic mucosa (fig 2D). Paneth cells were stained by the antibody for lysozyme (fig 2E). The location of Paneth cells in Cdx1 transgenic mouse stomach was different from that in

wild-type mouse intestine in which Paneth cells are located in the base of crypts.

To clarify the character of goblet cells, we stained intestinal metaplastic mucosa with Alcian blue. No Alcian blue staining (pH 2.5) was observed in the gastric mucosa of wild-type mice (not shown). In the gastric mucosa of 120 day old Cdx1 transgenic mice, Alcian blue (pH 2.5) stained goblet cells in the zymogenic zone (fig 3A). HID-Alcian blue staining (pH 2.5) showed that metaplastic glands were composed of mainly sulphomucin secreting goblet cells and a few sialomucin secreting goblet cells (fig 3B). To further characterise intestinal metaplasia, sections were stained for alkaline phosphatase (ALP). No ALP activity was observed in the gastric mucosa of wild-type mice (not shown). ALP activity was recognised on the surface area of intestinal metaplastic mucosa in Cdx1 transgenic mice (fig 3C).

We also examined the appearance of intestine specific enteroendocrine cells. Glicentin expressing cells were not observed in the gastric mucosa of wild-type mice (not shown). However, glicentin expressing enteroendocrine cells appeared in the metaplastic mucosa of 120 day old Cdx1 transgenic mice (fig 3D). We also found secretin or serotonin positive enteroendocrine cells but not cholecystokinin positive enteroendocrine cells in Cdx1 transgenic mouse stomach

Figure 3 (A) Intestinal metaplastic mucosa of 120 day old Cdx1 transgenic mouse stomach contained goblet cells stained blue by Alcian blue at pH 2.5. (B) Intestinal metaplastic mucosa of 120 day old Cdx1 transgenic mouse was covered by intestinal metaplasia composed of mainly sulphomucin (black) containing goblet cells and a few sialomucin containing goblet cells (blue), stained by high iron diamine-Alcian blue at pH 2.5. (C) Alkaline phosphatase activity was demonstrated by a red reaction product in Cdx1 transgenic mouse stomach at 120 days. (D) Glycintin enteroendocrine cells were seen in intestinal metaplastic mucosa of Cdx1 transgenic mouse stomach at 120 days. The inset is a magnified view with glycintin cells indicated by arrows. (E) Cdx2 was not expressed in intestinal metaplastic mucosa of 120 day old Cdx1 transgenic mouse stomach. (F) Cdx1 was not expressed in intestinal metaplastic mucosa of 120 day old Cdx2 transgenic mouse stomach. (G) Intestine specific gene expression in intestinal metaplastic mucosa of Cdx1 transgenic mice. Polymerase chain reaction products were derived from cDNAs reverse transcribed from RNAs of wild-type mouse glandular stomach (WT), wild-type mouse small intestine (SI), and Cdx1 transgenic mouse glandular stomach (TG).

(unpublished data). These results indicate that intestinal metaplastic mucosa is made up of all four cell lineages (absorptive enterocytes, goblet, enteroendocrine, and Paneth cells).

To determine if Cdx2 is involved in the formation of intestinal metaplasia in Cdx1 transgenic mice, we stained intestinal metaplastic mucosa with antibody for Cdx2. Cdx1 induced intestinal metaplastic mucosa did not express Cdx2 (fig 3E). Furthermore, Cdx2 induced intestinal metaplastic mucosa did not express Cdx1 (fig 3F). RT-PCR also showed that Cdx2 was not expressed in Cdx1 transgenic mouse stomach and Cdx1 was not expressed in Cdx2 transgenic mouse stomach (not shown).

To determine induction of intestine specific gene expression, we examined whether sucrase-isomaltase, villin, Muc2, Tff3, or cryptdin 1 was expressed in intestinal metaplastic mucosa using RT-PCR. All of these genes were expressed in Cdx1 transgenic mouse stomach and wild-type mouse intestine but not in wild-type mouse stomach (fig 3G).

To determine if Cdx1 transgenic fundic mucosa has gastric epithelial characteristics, we stained transgenic gastric specimens with antibodies for gastric mucosal markers in addition to H^+/K^+ -ATPase. The antibody for gastric mucin, MUC5AC, which is characteristic of gastric foveolar cells, diffusely stained wild-type mouse gastric mucosa (fig 4A). However, we did not observe obvious MUC5AC staining in the foveolar

Figure 4 Immunohistochemical staining for MUC5AC and pepsinogen C. (A) Foveolar-type gastric mucin (MUC5AC) was diffusely positive in 120 day old wild-type mouse gastric foveolar cells. (B) MUC5AC was rare in gastric mucosa of Cdx1 transgenic mice at 120 days. (C) Pepsinogen C was expressed in chief cells of 120 day old wild-type mouse stomach. (D) Pepsinogen C was not stained in Cdx1 transgenic mouse stomach at 120 days.

regions in intestinal metaplastic mucosa (fig 4B). Pepsinogen C was expressed in chief cells of wild-type mouse stomach (fig 4C) but was not detected in transgenic mouse stomach (fig 4D).

Finally, we measured the proliferative zone by PCNA staining in intestinal metaplastic mucosa of 50 week old mice (350 days). Only a small number of cells in the proliferative zone were PCNA positive in wild-type gastric mucosa (fig 5A).

Figure 5 Immunohistochemical staining for proliferating cell nuclear antigen (PCNA) in the fundic mucosa of wild-type, Cdx1, and Cdx2 transgenic mouse stomachs at the age of 50 weeks (350 days). Antibody for PCNA stained a large number of cells in intestinal metaplastic mucosa of Cdx1 transgenic mouse stomach (B) compared with the fundic mucosa of wild-type mouse stomach (A) or intestinal metaplastic mucosa of Cdx2 transgenic mouse stomach (C). (D) Ratio of the thickness of the proliferative zone to that of the gastric mucosa in wild-type, Cdx1, and Cdx2 transgenic mouse stomachs at the age of 50 weeks (350 days). Differences in the ratio between Cdx1 and Cdx2 mice or Cdx1 and wild-type mice were statistically significant (Student's *t* test, $p < 0.01$). Data are expressed as mean (SD). (E) Thickness of the fundic mucosa of wild-type, Cdx1, and Cdx2 transgenic mouse stomachs at the age of 50 weeks (350 days). The fundic mucosa of the Cdx1 transgenic mouse stomach was significantly thicker than that of wild-type or Cdx2 transgenic mouse stomach (Student's *t* test, $p < 0.01$). Data are expressed as mean (SD).

PCNA positive cells were widely scattered in Cdx1 intestinal metaplastic mucosa (fig 5B) while PCNA positive cells were in the base of the crypt of Cdx2 induced intestinal metaplastic mucosa (fig 5C). The proliferative zone markedly expanded in Cdx1 induced intestinal metaplastic mucosa (fig 5B). However, cells with ALP activity in the surface area were not stained by PCNA antibody (not shown). The ratio of the thickness of the proliferative zone to that of the gastric mucosa was mean 18 (SD 7)% in wild-type mice, 82 (7)% in Cdx1 transgenic mice, and 31 (11)% in Cdx2 transgenic mice (fig 5D). The gastric mucosa of the Cdx1 transgenic mice was thicker than that of wild-type mice or Cdx2 transgenic mice (fig 5A–C). The thickness of the gastric mucosa in Cdx1 transgenic mice was mean 865 (SD 45) μ m while those in wild-type mice and Cdx2 transgenic mice were 284 (23) μ m and 426 (40) μ m, respectively (fig 5E).

DISCUSSION

We established transgenic mice expressing Cdx1 in the gastric mucosa to analyse the relation between Cdx1 protein expression and intestinal metaplastic changes, and investigated differences in the roles of Cdx1 and Cdx2 in intestinal metaplasia. Remarkably, expression of a single gene, Cdx1, without expression of Cdx2, completely transformed gastric mucosa into intestinal metaplasia, indicating that Cdx1 has an essential role as a transcription factor in intestinal metaplasia.

We used the promoter of the H^+/K^+ -ATPase β subunit gene to express Cdx1 or Cdx2 specifically in gastric mucosa. Administration of the antiherpetic drug ganciclovir to transgenic mice, in which the promoter of the H^+/K^+ -ATPase β subunit gene was used to target expression of herpes simplex virus 1 thymidine kinase (HSV-1-tk) to parietal cells, caused rapid and specific ablation of parietal cells.²⁰ Parietal cell ablation also led to loss of other gastric epithelial cells (chief and mucus producing cells) that were not expression sites of the HSV-1-tk suicide gene.²⁰ Administration of the toxin to transgenic mice, in which the promoter of the H^+/K^+ -ATPase β subunit gene was used to direct expression of an attenuated diphtheria toxin A subunit in the parietal cell lineage, caused not only loss of parietal cells but also mucous neck and chief cells.²¹ Using the H^+/K^+ -ATPase β subunit gene promoter to express Cdx1 or Cdx2 specifically in gastric mucosa, expression of Cdx1 or Cdx2 in parietal cells may have affected the formation of mucous cells and chief cells and completely changed gastric mucosa into intestinal metaplastic mucosa.

The number of intestinal-type glands that expressed only Cdx1 with complete loss of H^+/K^+ -ATPase increased gradually until 120 days after birth, indicating that each gland gradually changed from gastric-type glands to intestinal-type glands. Finally, the gastric mucosa of Cdx1 transgenic mice was completely replaced by intestinal metaplastic mucosa. Mouse intestinal epithelium contains four principal terminally differentiated cell types: absorptive enterocytes, goblet, enteroendocrine, and Paneth cells, all of which were seen in intestinal metaplastic mucosa of Cdx1 transgenic mice. Enterocytes, goblet, and enteroendocrine cells differentiate and mature during normal migration that takes them from the crypt to the apex of the villus.^{22–24} On the other hand, Paneth cells are in the base of the crypt.²⁵ Both Cdx1 and Cdx2 have gradients of expression in the crypt-villus axis.⁷ Cdx1 is primarily in the crypt and Cdx2 is primarily in the villus. Absorptive enterocytes, goblet, and enteroendocrine cells are in the villus, which expresses Cdx2 but not Cdx1. However, in the Cdx1 transgenic mice, Cdx1 changed gastric mucosal cells to enterocytes, goblet, and enteroendocrine cells, indicating that Cdx1 can also induce differentiation of these three cell lineages without Cdx2 expression because

Cdx1 did not induce Cdx2 in the Cdx1 transgenic mouse stomach. Soubeyran *et al* reported that expression of Cdx1 in IEC-6 cells induced markers of enterocyte differentiation, aminopeptidase N, and villin.²⁶ Our results indicate that Cdx1 can induce differentiation of not only enterocytes but also enteroendocrine, goblet, and Paneth cells, even in gastric mucosal cells by expressing Cdx1.

The nature of intestinal metaplastic mucosa in Cdx1 transgenic mice was also determined by gene expression analysis in addition to histological examination. *Tff3* and *Muc2* genes, which are normally expressed in goblet cells, were expressed in Cdx1 induced intestinal metaplastic mucosa. *Sucrase-isomaltase* and *villin* genes, which are expressed in absorptive enterocytes, were also recognised in metaplastic mucosa. In the small intestine, a vertical gradient of *sucrase-isomaltase* gene expression was observed along the crypt-villus axis with a maximal level in the lower two thirds of the villi and no expression in crypts.^{27–29} Cdx2 protein was detected mainly in villus compartments^{7 17 30} and stimulates differentiation and expression of *sucrase-isomaltase*³¹ whereas Cdx1 protein is found mainly in the crypt compartment. However, our results indicate that Cdx1 can activate *sucrase-isomaltase* gene expression in intestinal metaplastic mucosa with ectopic expression of the *Cdx1* gene. The *cryptdin 1* gene, which is expressed in Paneth cells, was also expressed in the metaplastic mucosa of Cdx1 transgenic mice. Cdx1 expression in IEC-6 cells induced phenotypic changes characteristic of differentiating enterocytes.²⁶ However, Cdx1 has not been reported to induce differentiation of Paneth cells. Cdx1 transgenic mice clearly indicated that Cdx1 could differentiate Paneth cells expressing cryptdin 1 and lysozyme.

Cdx2 expressing transgenic mouse stomach produced pseudopyloric gland metaplasia while Cdx1 expressing transgenic mouse stomach did not. These results indicate that pseudopyloric gland metaplasia in human intestinal metaplasia may be related to Cdx2 expression rather than Cdx1 expression. On the other hand, Paneth cells, which were not seen in the Cdx2 transgenic mouse stomach,¹⁴ are recognised in the upper portion of Cdx1 induced intestinal metaplasia. Expression of EphB receptor is essential for correct positioning of epithelial cells along the crypt/villus axis.³² Paneth cells express high levels of EphB3 receptor.³² In the adult EphB3 null mice, Paneth cells do not follow their downward migratory path but were randomly distributed throughout the crypt and villus.³² The reason why Paneth cells are not confined to the base of crypts in our Cdx1 induced intestinal metaplastic mucosa may be related to expression of EphB3.

PCNA positive cells were widely distributed in Cdx1 expressing intestinal metaplastic mucosa, indicating that the proliferating zone was expanded in intestinal metaplastic mucosa by Cdx1 compared with Cdx2 expressing intestinal metaplastic mucosa and wild-type mouse gastric mucosa. PCNA positive cells in Cdx2 induced intestinal metaplastic mucosa were in the base of the crypt of metaplastic mucosa (fig 5C), similar to the location of PCNA positive proliferative cells in normal intestine. Several potential binding sites common to Cdx1 and Cdx2 were reported to be in the 5' flanking region of the human *PCNA* gene.³³ Cdx1 transactivated human *PCNA* gene promoter activity in a colorectal carcinoma cell line while Cdx2 did not.³³ These results may explain part of our findings that Cdx1 expressing intestinal metaplastic mucosa was diffusely PCNA positive and Cdx2 was not. Furthermore, intestinal metaplastic mucosa of Cdx1 transgenic mice was thicker than that of wild-type or Cdx2 transgenic mice. Soubeyran *et al* reported that expression of Cdx1 in IEC-6 cells induced anchorage independent growth in soft agar and tumour formation in nude mice.³⁴

Overexpression of Cdx1, as well as many other Hox genes, led to oncogenic transformation of cultured fibroblasts.³⁵ Moreover, ectopic expression of Cdx1 coincides with the development of metaplasia in gastric and oesophagus cancers.⁸ Taken together, the thick intestinal metaplastic mucosa with widely an expanded proliferative zone in Cdx1 transgenic mice might lead to changes that would be consistent with preneoplastic lesions.

In summary, Cdx1 and Cdx2 direct independent programmes to induce intestinal metaplasia with different differentiation, structure, and proliferation. Cdx1 and Cdx2 transgenic mice are very useful for clarifying differences in the roles of Cdx1 and Cdx2 in intestinal metaplasia.

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REFERENCES

- Parkin DM, Pisani P, Ferlay J. Estimates of the worldwide incidence of 25 major cancers in 1990. *Int J Cancer* 1999;**80**:827–41.
- Lauren P. The two histological main types of gastric carcinoma: diffuse and so-called intestinal-type carcinoma. *Acta Pathol Microbiol Scand* 1965;**64**:31–49.
- Correa P. A human model of gastric carcinogenesis. *Cancer Res* 1988;**48**:3554–60.
- Correa P. Human gastric carcinogenesis: a multistep and multifactorial process—First American Cancer Society Award Lecture on Cancer Epidemiology and Prevention. *Cancer Res* 1992;**52**:6735–40.
- Correa PSY. Phenotypic and genotypic events in gastric carcinogenesis. *Cancer Res* 1994;**54**(suppl):1941s–3.
- Meyer BI, Gruss P. Mouse cdx-1 expression during gastrulation. *Development* 1993;**117**:191–203.
- Silberg DG, Swain GB, Suh ER, et al. Cdx1 and Cdx2 expression during intestinal development. *Gastroenterology* 2000;**119**:961–71.
- Silberg DG, Furth EE, Taylor JK, et al. CDX1 protein expression in normal, metaplastic, and neoplastic human alimentary tract epithelium. *Gastroenterology* 1997;**113**:478–86.
- Mizoshita T, Inada K, Tsukamoto T, et al. Expression of Cdx1 and Cdx2 mRNAs and relevance of this expression to differentiation in human gastrointestinal mucosa—with special emphasis on participation in intestinal metaplasia of the human stomach. *Gastric Cancer* 2001;**4**:185–91.
- Eda A, Osawa H, Yanaka I, et al. Expression of homeobox gene CDX2 precedes that of CDX1 during the progression of intestinal metaplasia. *J Gastroenterol* 2002;**37**:94–100.
- Satoh K, Mutoh H, Eda A, et al. Aberrant expression of CDX2 in the gastric mucosa with and without intestinal metaplasia: effect of eradication of *Helicobacter pylori*. *Helicobacter* 2002;**7**:192–8.
- Bai YQ, Yamamoto H, Akiyama Y, et al. Ectopic expression of homeodomain protein CDX2 in intestinal metaplasia and carcinomas of the stomach. *Cancer Lett* 2002;**176**:47–55.
- Almeida R, Silva E, Santos-Silva F, et al. Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. *J Pathol* 2003;**199**:36–40.
- Mutoh H, Hakamata Y, Sato K, et al. Conversion of gastric mucosa to intestinal metaplasia in Cdx2-expressing transgenic mice. *Biochem Biophys Res Commun* 2002;**294**:470–9.
- Silberg DG, Sullivan J, Kang E, et al. Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. *Gastroenterology* 2002;**122**:689–96.
- Duprey P, Chowdhury K, Dressler GR, et al. A mouse gene homologous to the *Drosophila* gene *caudal* is expressed in epithelial cells from the embryonic intestine. *Genes Dev* 1988;**2**:1647–54.
- James R, Erler T, Kazenwadel J. Structure of the murine homeobox gene *cdx-2*. Expression in embryonic and adult intestinal epithelium. *J Biol Chem* 1994;**269**:15229–37.
- Subramanian V, Meyer B, Evans GS. The murine Cdx1 gene product localises to the proliferative compartment in the developing and regenerating intestinal epithelium. *Differentiation* 1998;**64**:11–18.
- Hogan B, Beddington R, Costantini F, et al. *Manipulating the mouse embryo: a laboratory manual*, 2nd edn. New York: Cold Spring Harbor Laboratory Press, 1994.
- Canfield V, West AB, Goldenring JR, et al. Genetic ablation of parietal cells in transgenic mice: A new model for analyzing cell lineage relationships in the gastric mucosa. *Proc Natl Acad Sci USA* 1996;**93**:2431–5.
- Li Q, Karam SM, Gordon JL. Diphtheria toxin-mediated ablation of parietal cells in the stomach of transgenic mice. *J Biol Chem* 1996;**271**:3671–6.
- Cheng H, Leblond CP. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. I. Columnar cell. *Am J Anat* 1974;**141**:461–79.
- Potten CS, Allen TD. Ultrastructure of cell loss in intestinal mucosa. *J Ultrastruct Res* 1977;**60**:272–7.
- Wright NA, Irwin M. The kinetics of villus cell populations in the mouse small intestine. I. Normal villi: the steady state requirement. *Cell Tissue Kinet* 1982;**15**:595–609.
- Cheng H. Origin, differentiation and renewal of the four main epithelial cell types in the mouse small intestine. IV. Paneth cells. *Am J Anat* 1974;**141**:521–35.
- Soubeyran P, Andre F, Lissitzky JC, et al. Cdx1 promotes differentiation in a rat intestinal epithelial cell line. *Gastroenterology* 1999;**117**:1326–38.
- Traber PG. Regulation of sucrase-isomaltase gene expression along the crypt-villus axis of rat small intestine. *Biochem Biophys Res Commun* 1990;**173**:765–73.
- Traber PG, Yu L, Wu GD, et al. Sucrase-isomaltase gene expression along crypt-villus axis of human small intestine is regulated at level of mRNA abundance. *Am J Physiol* 1992;**262**:G123–30.
- Rings EH, Krasinski SD, van Beers EH, et al. Restriction of lactase gene expression along the proximal-to-distal axis of rat small intestine occurs during postnatal development. *Gastroenterology* 1994;**106**:1223–32.
- Rings EH, Boudreau F, Taylor JK, et al. Phosphorylation of the serine 60 residue within the Cdx2 activation domain mediates its transactivation capacity. *Gastroenterology* 2001;**121**:1437–50.
- Suh E, Traber PG. An intestine-specific homeobox gene regulates proliferation and differentiation. *Mol Cell Biol* 1996;**16**:619–25.
- Battle E, Henderson JT, Beghtel H, et al. Beta-catenin and TCF mediate cell positioning in the intestinal epithelium by controlling the expression of EphB/ephrinB. *Cell* 2002;**111**:251–63.
- Oh EJ, Park JH, Cho M, et al. The caudal-related homeodomain protein CDX1 activates proliferating cell nuclear antigen expression in hepatocellular and colorectal carcinoma cells. *Int J Oncol* 2002;**20**:23–9.
- Soubeyran P, Haglund K, Garcia S, et al. Homeobox gene Cdx1 regulates Ras, Rho and PI3 kinase pathways leading to transformation and tumorigenesis of intestinal epithelial cells. *Oncogene* 2001;**20**:4180–7.
- Maulbecker CC, Gruss P. The oncogenic potential of deregulated homeobox genes. *Cell Growth Differ* 1993;**4**:431–41.

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