Helicobacter pylori induces gastric mucosal intestinal metaplasia through the inhibition of interleukin-4-mediated HMG box protein Sox2 expression

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Helicobacter pylori induces gastric mucosal intestinal metaplasia through the inhibition of interleukin-4-mediated HMG box protein Sox2 expression. Am J Physiol Gastrointest Liver Physiol 297: G312–G322, 2009. First published June 11, 2009; doi:10.1152/ajpgi.00518.2007.—Helicobacter pylori is a major cause of the transdifferentiation into intestinal metaplasia that may develop gastric cancer. However, the molecular pathogenesis of this transdifferentiation is poorly understood. A SRY-related HMG box protein Sox2 is an essential transcription factor of organ development in brain, lung, and stomach. Our aim of this study was to investigate the mechanism responsible for regulation of Sox2 in host Th1-dominant response to H. pylori. Sox2 protein was immunohistochemically expressed in both human oxyntic and pyloric glands with H. pylori infection, but not in intestinal metaplasia. Western immunoblotting of gastric epithelial cell lines showed that IL-4, a Th2-related cytokine, dose dependently enhanced Sox2 expression among H. pylori infection-mediated cytokines. Small changes of Sox2 expression were observed after each treatment with IFN-γ, IL-1β, or TNF-α. IL-4 mediated Sox2 induction was suppressed by the inhibition of STAT6 activation with STAT6 RNA interference, and electrophoretic mobility shift assay indicated that activation of the Sox2 promoter by IL-4 occurred through the action of STAT6. Furthermore, H. pylori and IFN-γ inhibited the phosphorylation of STAT6, resulting in the suppression of IL-4-mediated Sox2 expression. Immunohistochemical analyses showed significantly the suppressed STAT6 activity in H. pylori-infected human gastric mucosa. Additionally, downregulation of Sox2 by knockdown experiments led to intestinal phenotype with expressions of Cdx2 and MUC2. These results suggest that H. pylori and IFN-γ interfere with the differentiation into oxyntic and pyloric glands by the downregulation of Sox2 on IL-4/STAT6 signaling, which may contribute to the transdifferentiation into intestinal metaplasia.

transdifferentiation; STAT6 signaling; Cdx2

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ulcer at Tohoku University Hospital (Sendai, Japan). Nonneoplastic gastric mucosa distant from carcinomas was collected. In addition, biopsy specimens after H. pylori eradication were also obtained from six patients among them. The presence of H. pylori infection was confirmed by at least one positive result of three different methods for determining the H. pylori status: histology, rapid urease test, \(^{13}\)C urea breath test. In addition, to confirm H. pylori eradication, H. pylori negativity was defined by all of these three different tests 2 mo after eradication. This study was conducted with an approval from the Ethical Review Board of Tohoku University and examined after obtaining of informed consent from the subjects.

**Cell culture.** AGS, Caco-2, and HT29 were obtained from the American Type Culture Collection (ATCC, Manassas, VA). NUGC4, KATOIII, GCIY, and THP-1 were obtained from Cell Resource Center for Biomedical Research, Tohoku University (Sendai, Japan). MKN45 and MKN28 were purchased from the Health Science Research Resources Bank (Osaka, Japan). AGS was cultured in F-12 broth (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS). The other cell lines were cultured in RPMI-1640 (Invitrogen) with 10% FBS. All cell lines were incubated at 37°C in a 5% CO\(_2\) atmosphere for experiment.

**Growth of bacteria and conditions of infection.** H. pylori (cagA+ strain 43504; ATCC) was grown on Skirrow plates (Nissui, Tokyo, Japan) and incubated at 37°C in a microaerophilic condition (10% CO\(_2\), 5% O\(_2\), and 85% N\(_2\)). After an overnight incubation on the plates, bacteria were cultured in a Brucella broth (Invitrogen) supplemented with 10% FBS for 2 days. Bacterial numbers were standardized by optical density measurement at 600 nm [OD600, 1.5 = 3.6 \times 10^6 colony forming units (cfu)/ml] according to the previous report (23).

**Immunohistochemistry.** Tissue samples were fixed in 10% buffered formalin and embedded in paraffin. Serial sections cut from paraffin blocks (3 \(\mu\)m) were dewaxed. After the inhibition of endogenous peroxidase activity, antigen retrieval was carried out by microwaving the sections in 10 mmol/l citrate buffer for 30 min. For first antibodies, we applied anti-Sox2 1:2,000 (Chemicon, Temecula, CA), anti-Cdx2 1:80 (BioGenex, San Ramon, CA), anti-MUC5AC 1:100 (Novocastra Laboratories, Newcastle, UK), anti-MUC2 1:100 (Novocastra Laboratories), anti-Ki-67 1:200 (DakoCytomation, Glostrup, Denmark), and anti-phosphorylated-signal transducers and activator of transcription 6 (pSTAT6) 1:300 (Cell Signaling Technology, Beverly, MA) and incubated sections overnight at 4°C. After the application of appropriate secondary antibodies for 30 min, bindings were visualized with a mixture of 0.03% dianaminobenzidine and 0.05% hydrogen peroxide in 0.05 M Tris HCl buffer. Sections were counterstained with hematoxylin in the standard fashion.

The pSTAT6-positive ratio was calculated by counting pSTAT6-positive epithelial cells in the entire gastric pits longitudinally sectioned and representing them as a percentage of the total epithelial cells in a gastric pit. At least 10 well-oriented gastric pits (more than 500 epithelial cells) were examined for each specimen. The count was performed in high-power view \((\times 100)\) by two observers (S. Asonuma and A. Imatani) independently. Only nuclei stained unequivocally positive epithelial cells in the entire gastric pits longitudinally sectioned were considered positive.

**Stimulation with cytokines and H. pylori.** Human gastric epithelial cells, AGS, and MKN45 were stimulated with IFN-\(\gamma\) (R&D Systems, Minneapolis, MN), IL-1B (Roche, Indianapolis, IN), TNF-\(\alpha\) (Roche), and IL-4 (PeproTech, London, UK), respectively, according to each procedure. Another set of stimulation with IL-4 (50 ng/ml) for 8 h was performed in these cell lines after the pretreatment with H. pylori or IFN-\(\gamma\) for 24 h.

**Western blot analysis.** Proteins were extracted by using a lysis buffer that consisted of 0.05 M Tris·HCl (pH 7.5), 0.15 M NaCl, 1%
deoxycholic acid, 1% Triton X-100, 0.1% SDS, Complete Mini (protease inhibitor cocktail tablet, Roche) and phosphatase inhibitors. The lysate was centrifuged and the supernatant was stored at -80°C.

An equal volume of protein (50 μg) for each sample was separated by 10% Tris-glycine polyacrylamide gel (Bio-Rad; Hercules, CA) electrophoresis, and transferred to polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). After blocking, membranes were incubated with each dilution of primary antibodies (Anti-Sox2; 1:4,000; Anti-Cdx2; 1:2,500; Anti-pSTAT6; 1:2,500; Anti-STAT6; 1:2,500) for overnight at 4°C. After washing with 0.05% Tween 20 in Tris-buffered saline, membranes were incubated with each secondary antibody for 1 h. Protein bands were detected.

Fig. 2. Representative immunohistochemical sections of Sox2, MUC5AC, Cdx2 and MUC2 in H. pylori-infected human pyloric mucosa and intestinal metaplasia. In pyloric mucosa (left), Sox2 is expressed in both foveolar and pyloric glandular cells. In the region of transitional differentiation from pyloric gland to intestinal metaplasia (middle), glands stained with both Sox2 and MUC5AC do not express Cdx2 and MUC2. In intestinal metaplasia (right), neither Sox2 nor MUC5AC are expressed. Inversely Cdx2 and MUC2 are expressed. ▲, Paneth cells. Original magnification: ×100.
with ECL advance reagent (Amersham Biosciences, Little Chalfont, Buckinghamshire, UK) and were visualized by use of a luminoimage analyzer (LAS1000plus; Fujifilm, Tokyo, Japan). Band density was assessed semiquantitatively by utilizing computer-assisted densitometry (Scion Image 1.62c) (Scion, Frederick, MD). The examined protein/β-actin protein ratio of the immunoreactive area was calculated by densitometry.

Total RNA isolation and reverse transcription-polymerase chain reaction. Total RNA was isolated with TRIzol reagent (Invitrogen) according to the manufacturer’s recommendations. The isolated RNA was used for cDNA synthesis with a SuperScript III transcriptase (Invitrogen). Semiquantitative touchdown polymerase chain reaction (PCR) was carried out. Reaction mixtures and cycling conditions were described previously (32). Sense and antisense primers used are listed in Table 1.

Nuclear protein extraction and electrophoretic mobility shift assay. Nuclear protein extracts were prepared from MKN45 cells stimulated with IL-4 (50 ng/ml) for 3 h by using a Celllytic NuCLEAR Extraction Kit (Sigma Chemical, St. Louis, MO) as recommended by the supplier.

EMSA was performed as previously described (27). An oligonucleotide probe containing STAT6 binding region specific for Sox2 (5′-acctacctcctctcgaaagctgt-3′; from −282 to −258) was labeled with [γ-32P]ATP by use of T4 polynucleotide kinase (BioLabs, Beverly, MA). The nuclear protein extract (5 μg) was incubated with the labeled oligonucleotide probe for 20 min at room temperature and electrophoresed through 6% polyacrylamide gels with 0.5× Tris-borate-EDTA buffer. Gels were dried and visualized by autoradiography. The nucleoprotein complexes were visualized by use of a luminoimage analyzer (Scion Image 1.62c). The examined protein band density in each condition was calculated by densitometry.

To determine whether Sox2 expression would be altered by IFN-γ, IL-1β, TNF-α, and IL-4, mediated by H. pylori infection, Western blots analyses were performed in both AGS and MKN45 cells. No change in Sox2 expression was found 24 h after the stimulation with IFN-γ in the both cells (Fig. 4A). Similarly, neither IL-1β nor TNF-α affected Sox2 expression (Fig. 4, B and C). When stimulated with IL-4, Sox2 expression was enhanced dose dependently in both AGS and MKN45 cells. The level of Sox2 expression in band density was twofold on AGS cells and 2.5-fold on MKN45 cells stimulated with 50 ng/ml IL-4, compared with nontreated cells (Fig. 4D).

Requirement of STAT6 in the regulation of Sox2 gene expression by IL-4. We assessed the activation of STAT6 induced by IL-4 in MKN45 cells. Increased expression of both phosphorylated STAT6 and Sox2 was observed dose dependently with 24 h of IL-4 stimulation (Fig. 5A). An induction of phosphorylated STAT6 was observed within 1 h upon IL-4 stimulation (50 ng/ml). Sox2 expression was enhanced time dependently by 3 h after the IL-4 stimulation. Similar results were observed on AGS cells (data not shown).

To further confirm that IL-4-mediated STAT6 signaling regulated Sox2 expression, knockdown experiments using small interference RNA (siRNA) oligonucleotides specific for human STAT6 and Sox2 (ON-TARGET plus SMARTpool) from Dharmacon (Lafayette, CO). Mock siRNA oligonucleotides (siCONTROL Non-Targeting siRNA Pool, Dharmacon) were used as a negative control.

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STAT6 siRNA were performed in AGS cells. In Fig. 5B, the transfection of 50 nM STAT6 siRNA abrogated the translation of STAT6 protein, resulting in the decrease of phosphorylated STAT6 mediated by IL-4 treatment. This reduction of STAT6 activation led to the inhibition of Sox2 expression. The transfection of mock siRNA did not block the translation of STAT6 protein.

Identification of a STAT6 binding site in the Sox2 promoter.
We have investigated whether a putative STAT6 binding site exists in the Sox2 promoter. Computer-based analysis of the −632 to +44 of the human Sox2 promoter (GenBank accession number: NW 921807) using MatInspector (Genomatix, Munchen, Germany) identified a putative STAT6 binding site at position −276 to −267 position (Fig. 6A). The sequence motif of STAT6 binding site is perfectly conserved among human, mouse (GenBank accession number: NT 162143), and rat (GenBank accession number: NW 047625) (Fig. 6B). Therefore, EMSA experiments were conducted to confirm whether the site could interact with STAT6 proteins with the use of nuclear extracts prepared from IL-4 treated MKN45 cells. In Fig. 6C, DNA binding indicated by an arrow was increased in IL-4-treated cells (lane 2) compared with that in nontreated cells (lane 1). The specificity of the STAT6 binding was confirmed by the addition of cold competitor oligonucleotides (lane 3) and by the ability of antibody specific for STAT6 to block the DNA complex formation (lane 4).

Fig. 4. Influences of H. pylori related cytokines with various concentrations of IFN-γ (A), IL-1β (B), TNF-α (C), and IL-4 (D) on expression levels of Sox2 protein. Whole cell protein was extracted from AGS and MKN45 cells after 24 h stimulation with each cytokine. β-Actin expression was used as an internal loading control. Densitometric analysis for the band density of Sox2 over β-actin protein was performed. Graphs represent means ± SD of 3 separate experiments. Error bars represent SD. *P < 0.05 relative to control cells.
Suppressive effect of *H. pylori* and IFN-γ on the expressions of Sox2 and MUC5AC. Some studies reported that *H. pylori* and IFN-γ suppress IL-4 mediated STAT6 signaling (6, 41). To determine whether *H. pylori* and IFN-γ inhibited IL-4 mediated Sox2 expression, Western blots and RT-PCR analyses were performed in both AGS and MKN45 cells.

When IL-4 (50 ng/ml) posttreated MKN45 cells were cocultured with *H. pylori*, as expected, the expression level of Sox2 was decreased through the abrogation of STAT6 phosphorylation to the level reaching 53% at 5.0 × 10^5 cfu/ml *H. pylori* infection (Fig. 7A). The mRNA level of MUC5AC that is upregulated by Sox2 was dose dependently decreased in *H. pylori* infection (Fig. 7B). Similar results were found using AGS cells (data not shown). As well as the results from *H. pylori* stimulation, IFN-γ, a Th1-related cytokine induced by *H. pylori* infection, strikingly diminished the expression levels of Sox2 (Fig. 8A) and MUC5AC (Fig. 8B). These results demonstrated that both *H. pylori* infection and IFN-γ stimulation reduced IL-4-mediated Sox2 expression through STAT6 signaling.

Inhibition of phosphorylation of STAT6 in *H. pylori*-infected human gastric mucosa. To confirm whether *H. pylori* infection inhibits the phosphorylation of STAT6 in human gastric mucosa, the change of STAT6 activity before and after *H. pylori* eradication was evaluated by immunohistochemistry. In Fig. 9A, pSTAT6 staining was observed in the nuclei of both gastric epithelial cells and mononuclear cells in the *H. pylori*-infected and post eradicated gastric mucosa in all six cases. The pSTAT6-positive ratio was 53.3 ± 14.8% in *H. pylori*-infected epithelial cells; on the other hand, in post eradicated epithelial cells, the ratio was 74.2 ± 15.5% (Fig. 9B). The pSTAT6-positive ratio was significantly higher in the post eradicated cells than in the infected cells, suggesting that the phosphorylation of STAT6 was abrogated in *H. pylori*-infected epithelial cells and restored by the removal of *H. pylori* infection.

**Induction of Cdx2 and MUC2 by Sox2 siRNA interference.** To determine whether the downregulation of Sox2 led to intestinal phenotype in gastric epithelial cells, interference experiments by inserting Sox2 siRNA was performed. When AGS cells were transiently transfected with Sox2 siRNA for 48 h, Sox2 protein was minimally expressed compared with the control (17.3 ± 0.85%). Inhibition of Sox2 by the siRNA significantly increased Cdx2 expression reaching up to 2.2-fold level (Fig. 10A). In contrast, treatment of cells with mock siRNA did not alter Sox2 expression. In the levels of mRNA (Fig. 10B), MUC5AC expression was blocked following the Sox2 suppression. Cdx2 expression was induced, and MUC2 was also evoked by the Sox2 suppression, providing that downregulation of Sox2 expression led to intestinal phenotype in vitro.

**Discussion.** We have shown that Sox2 is regulated by IL-4 through STAT6 signaling pathway in gastric epithelial cells and that this regulation is suppressed with *H. pylori* infection and IFN-γ stimulation in relation to the transdifferentiation to intestinal metaplasia.

A HMG box protein Sox2 maintains the “stem” status in ES cells (5, 43) and is also associated with organ development of neural tissue and foregut-derived organs (38) including the stomach (20). In the human adult oxyntic mucosa, results of our immunohistochemical studies first demonstrated that Sox2 immunoreactivity was marked in the proliferative cell zone where stem cells exist and was sporadically present in foveolar and oxyntic glandular cells, where MUC5AC and pepsinogen are expressed, respectively. As Tsukamoto et al. (48) previously reported, Sox2 protein was expressed in both foveolar and glandular cells of pyloric mucosa. In vivo analysis showed that Sox2 protein transcriptionally regulates MUC5AC (25). In addition, the results of in situ analysis of the chicken embryo demonstrated that Sox2 protein regulates the expression of pepsinogen gene in the developing gut (20). Other in vitro analysis also demonstrated that overexpression of Sox2 induces pepsinogen gene in human embryonic kidney-derived cells (45). Taken together, Sox2 may be an essential gene to maintain the differentiation of oxyntic and pyloric glands as well as neural tissues.
H. pylori infection to gastric mucosa causes multifocal atrophic gastritis in both the antrum and body. Histopathologically, this gastritis is characterized by loss of oxyntic and pyloric glands and leads to oxyntic and pyloric metaplasia, that is, the replacement by an intestinal-type epithelium (7, 8). In this study, Sox2 protein was downregulated and inversely Cdx2 protein was emerged in proportion to the progression from pyloric glands to intestinal metaplasia in H. pylori-infected mucosa. One report also presented that the expression level of Sox2 was repressed even in the process from incomplete to complete intestinal metaplasia (48). Therefore, we addressed our study on the hypothesis that the downregulation of Sox2 expression in oxyntic and pyloric glands by host immune response to H. pylori leads to the transdifferentiation into intestinal metaplasia.

When the two gastric cell lines were stimulated by several cytokines associated with the immune response to H. pylori, unexpectedly Sox2 expression was not suppressed independently by IL-1β, TNF-α, and IFN-γ, which are thought to be secreted more in H. pylori-infected gastric mucosa. But we elucidated that IL-4 induced Sox2 expression in both two gastric cell lines time and dose dependently. A Th2-related cytokine IL-4 is an important regulator of the proliferation and differentiation of cells, especially lymphocytes, mediated by STAT6 phosphorylation (16). When we searched for the immune system-related sequences with MatInspector, a computer-based program, it revealed the STAT6 binding motif (TTCNNNNGAA) in the Sox2 promoter region from -276 to -267. Our results from STAT6 RNA interference and EMSA experiments for this motif showed that IL-4/STAT6 signaling pathway was one of the Sox2 expression...
inducible mechanisms in the gastric epithelium. There are other representative binding sites predicted on the computed analysis such as NF-κB and OCT (Fig. 6C). Therefore, it may be important to determine whether these binding sites also represent transcriptional function in the immune response to *H. pylori* infection.

We observed that STAT6 was activated in the epithelial cells of oxyntic glands, but a limitation of our study is that measurements of IL-4 in gastric mucosa were not available. Generally, it has been widely reported that *H. pylori* infection exhibits Th1 immune response characterized by recruitment of IFN-γ-expressing T cell and low levels of Th2 cells expressing IL-4 (22, 29, 42). Although it remains controversial whether *H. pylori* infection influences the level of IL-4 production, interestingly, IL-4 is potentially secreted even in the uninfected mucosa (21, 22, 34, 37, 42). Moreover, as an effect of IL-4 on the stomach, Merchant and colleagues (51) reported that IL-4 and INF-γ reciprocally influence on the secretion of both gastrin and somatostatin, which regulate acid secretion in the stomach and immune response to *H. pylori* infection.

In general, IL-4 promotes not only the differentiation of lymphocytes into Th2 phenotype and B cells (44) but also the differentiation of epithelial cells in some organs. For example,
some studies showed that IL-4 directly induced mucin gene expression and the differentiation of airway epithelial cells into goblet cell (9, 47, 49). Similarly, it was reported that IL-4- and IL-13-induced STAT6 activation was required to induce Trefoil factor family 3 secreted by mucin-producing colon epithelia (4). In Barrett’s esophagus characterized by the transdifferentiation from squamous to columnar epithelium, IL-4 mRNA level was increased fourfold compared with squamous esophageal mucosa with esophagitis (12). Corresponding with these studies, our results show that IL-4/STAT6 signaling may also contribute to the differentiation into oxyntic and pyloric glands of the stomach through the activation of Sox2.

In terms of STAT6 signaling activated by IL-4, current evidence shows that IFN-γ inhibits IL-4/STAT6 signaling in Th lymphocytes (18, 41) and airway epithelial cells (17), mediated by the downregulation of IL-4 receptor gene expression and the prevention of recruitment of STAT6 to IL-4 receptor signaling complex. In addition, Ceponis et al. (6) revealed in the gastric epithelial cell lines that H. pylori itself abrogated phosphorylation of STAT6 and inhibited the translocation of phosphorylated STAT6 into nucleus. In our study, as expected, both H. pylori itself and IFN-γ suppressed IL-4-induced Sox2 expression by the inhibition of IL-4/STAT6 signaling in the gastric epithelial cells. Also, our immunohistochemical study showed, in vivo, that the activation of STAT6 signaling was significantly restored after H. pylori eradication, that is, in the human gastric mucosa free from H. pylori. Therefore, our results show that both H. pylori infection and Th1-dominant host immune response blocks the Sox2 expression in the point of STAT6 signaling, resulting in the suppression of differentiation into oxyntic and pyloric glands, which might lead to gastric atrophy and intestinal metaplasia.

Finally, we confirmed with Sox2 siRNA experiments that Sox2 suppression induced Cdx2 expression in the gastric epithelial cells, involving in the inhibition of MUC5AC gene and the induction of MUC2 gene expression. Accordingly, it has been elucidated that Sox2 suppression could lead to the transdifferentiation into intestinal metaplasia in adult gastric

![Fig. 9](http://ajpgi.physiology.org/)

**Fig. 9. Inhibition of phosphorylation of STAT6 in H. pylori-infected human gastric mucosa.** *A*: immunohistochemistry of pSTAT6 expression in oxyntic glands on both *H. pylori*-infected and post-eradicated human gastric mucosa. Original magnification: ×100. *B*: the pSTAT6-positive ratio in both *H. pylori*-infected and post-eradicated gastric mucosa was calculated by counting pSTAT6-positive epithelial cells. The results are means ± SE of 6 patients. Error bars represent SD.

![Fig. 10](http://ajpgi.physiology.org/)

**Fig. 10. Effect of Sox2 siRNA interference on induction of intestinal phenotype in AGS cells.** *A*: Cdx2 protein expression was examined by Western blot after the addition of 50 nM Sox2 siRNA. Mock siRNA was used as a negative control. Densitometric analysis of Sox2 and Cdx2 proteins over β-actin protein. Graphs represent means ± SD of 3 separate experiments. Error bars represent SD. *P < 0.05 relative to control cells. B*: MUC5AC, Cdx2, and MUC2 mRNA levels were assessed by RT-PCR after the addition of 50 nM Sox2 siRNA. Mock siRNA was used as a negative control. RNAi, RNA interference.
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mucosa. In other words, Sox2 suppression by H. pylori infection and Th1 dominant host immune response may trigger Correa’s sequence. Indeed, Otsubo et al. (35) reported that Sox2 inhibits cell growth through cell-cycle and apoptosis in gastric cancer cells, suggesting that Sox2 suppression may be related to gastric carcinogenesis.

In E5 cells, recent studies have clarified that Sox2 expression is regulated to maintain the pluripotency under a complicated network with many genes (30, 30). Especially on the interaction with Cdx2, Sox2 was reported to mediate a negative loop on Cdx2 through Oct3/4, a member of the POU class of homeodomain protein (31, 33). But there is little information about a signal pathway between Sox2 and Cdx2 in gastric epithelium. Therefore, it is necessary to investigate what mechanism regulates the interaction between Sox2 and Cdx2 in adult differentiated gastric mucosa to clarify the transdifferentiation from oxyntic and pyloric glands to intestinal metaplasia.

In conclusion, we demonstrated both that H. pylori infection and IFN-γ inhibited Sox2 expression with the stimulation of IL-4 through STAT6 signaling pathway and that Sox2 suppression increased the expression of Cdx2. Accordingly, these data suggest that the downregulation of Sox2, which plays an essential role in the differentiation into oxyntic and pyloric glands, may promote intestinal metaplasia in H. pylori infection and Th1-dominant host immune response.

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