CDX1 Expression Induced by CagA-Expressing *Helicobacter pylori* Promotes Gastric Tumorigenesis

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Abstract

Intestinal-type gastric cancer often results from *Helicobacter pylori* infection through intestinal metaplasia, a transdifferentiated premalignant phenotype. Because *H. pylori* virulence factor CagA has been associated with aberrant expression of the transcription factor CDX1, which regulates intestinal differentiation, we explored its relationship with *H. pylori* infection and function during gastric carcinogenesis in normal gastric epithelial cells and gastric cancer cell lines. Infection of HFE 145 cells with CagA+ *H. pylori* increased expression of CDX1, as well as the epithelial-to-mesenchymal transition (EMT) markers Snail and Slug, increased invasion and migration, but those effects were not found in HFE 145 cells infected with CagA-deficient *H. pylori*. CDX1 overexpression increased expression of the intestinal markers Villin, sucrase isomaltase (SI), and MUC2, induced spheroid formation, and enhanced expression of the stem cell markers CD44, SOX2, Oct4, and Nanog, while CDX1 knockdown inhibited proliferation and intestinal stemness. Treatment of CDX1-expressing cells with metformin, an antidiabetic drug known to decrease the risk of gastric cancer, decreased expression of EMT and stemness markers, and reduced spheroid formation. In a murine xenograft model, combining metformin or shCDX1 with cisplatin reduced tumor growth, increased caspase-3 cleavage, and reduced expression of CD44 and MMP-9 to a greater degree than cisplatin alone. Patients with more advanced intestinal metaplasia staging exhibited higher CDX1 expression than those with earlier intestinal metaplasia staging (*P* = 0.039), and those with *H. pylori* tended to have more CDX1 expression than noninfected patients (*P* = 0.061). Finally, human tissue samples with higher CDX1 levels showed prominent CD44/SOX2 expression. Our findings indicate CagA+ *H. pylori*-induced CDX1 expression may enhance gastric cancer tumorigenesis and progression, and support therapeutic targeting of CDX1 in gastric cancer.

Implications: This study shows that CDX1 contributes to the tumorigenesis and progression of gastric cancer and suggests the potential of targeting CDX1 to treat this malignancy.

Introduction

Gastric cancer is classified as intestinal or diffuse gastric cancer (1). Intestinal-type gastric cancer, which accounts for more than half of all gastric cancer cases (2), arises in parallel with environmental factors, such as *Helicobacter pylori* infection and certain dietary conditions (3). Infection with *H. pylori* strains expressing the virulence factor Cytotoxin-associated gene A (CagA) is associated with greater gastric cancer risk than CagA-negative strains (4, 5). CagA is highly associated with development of intestinal metaplasia (intestinal metaplasia; refs. 6, 7), the precursor to intestinal-type gastric cancer in which the gastric epithelium transdifferentiates to display properties of intestinal epithelium. Several possible pathways, including interaction of CagA with E-cadherin and deregulation of the β-catenin signaling pathway, have been proposed to explain this pathologic change (8, 9). However, the mechanism by which CagA promotes transdifferentiation from gastric epithelium to enteroocyte-like epithelium remains unclear.

Caudal type homeobox 1 (CDX1) is a homeobox transcription factor that plays an important role in human intestinal development and maintenance (10). CDX1 is encoded by *CDX1*, a β-catenin–dependent gene transactivated by deregulated β-catenin, which can be triggered by CagA from *H. pylori* (11). Gene expression comparisons between *H. pylori*-infected gastric mucosa from Rhesus macaque and controls showed that *H. pylori*-infected gastric epithelia exhibited 1.59-fold (*P* = 0.004) increased CDX1 expression (GSE8749; Supplementary Fig. S1A; ref. 12). The expression of CD44 and Slug, which are considered to be associated with stemness and epithelial-
mesenchymal transition (EMT), also increased upon infection with *H. pylori*. Aberrantly expressed CDX1 has also been found to associate with development of Barrett esophagus and the presence of intestinal metaplasia, dysplasia, and gastric cancer in humans (13–16), although the biological role of dysregulated CDX1 has not been fully elucidated. Therefore, we explored the induction of CDX1 by *H. pylori* CagA and its function in gastric carcinogenesis. As the prior study showing that CagA increases CDX1 expression employed gastric cancer cells stably expressing CagA (11), we examined whether infection with CagA-expressing *H. pylori* has the same effect. We investigated CDX1's effects on dedifferentiation into stem-like cells and transdifferentiation into intestinal metaplasia by inducing CDX1 overexpression in gastric epithelial cells and by knocking down CDX1 in gastric cancer cells. We also examined tissues from patients with gastritis or gastric cancer with varying degrees of intestinal metaplasia to validate our *in vitro* findings. Furthermore, we evaluated the feasibility of using CDX1 as a potential target for chemoprevention or treatment for gastric cancer using murine xenograft models.

**Figure 1.** *H. pylori* infection induces CDX1 expression in gastric epithelial cells. **A,** Several gastric cancer (GC) cells display various CDX1 expression statuses. **B,** CDX1 expression was stronger in HFE 145 cells treated with CagA ÷ *H. pylori* than those infected with CagA ύ strain. Its expression measured with densitometry was higher in CagA ÷ *H. pylori*-infected HFE 145 cells. **C,** The expression of SOX2 increased significantly with WT *H. pylori* infection in immunofluorescence assay. **D,** Luciferase assays confirmed CagA-dependent CDX1 expression and putatively identified the potential binding site for CagA-associated factor. (Continued on the following page.)
Materials and Methods

Cell lines and reagents
HFE 145 normal human gastric epithelial cells were kindly provided by Dr. Hassan Ashktorab and Duane T. Smoot (Howard University, Washington, DC) and SNU1, SNU5, and SNU16, SNU216, SNU638, and SNU668 human gastric cancer cells were purchased from the Korean Cell Line Bank. AGS and MKN28, KATOIII, YCC-2, and NCI-N87 human gastric cancer cells were obtained from the ATCC. ATCC uses morphology, karyotyping, and PCR-based approaches to confirm the identity of human cell lines and to rule out both intra- and interspecies contamination. These include an assay to detect species specific variants of the cytochrome C oxidase I gene (COI analysis) to rule out interspecies contamination and short tandem repeat (STR) profiling to distinguish between individual human cell lines and rule out intraspecies contamination. All cells were maintained and cultured in monolayer or as spheroids as described previously. KCLB uses DNA fingerprinting analysis, species verification testing, Mycoplasma contamination testing, and viral contamination testing. Cancer cell lines were actively passaged for less than 6 months from the time that they were received from ATCC or KCLB.

Cisplatin was purchased from Donga ST, 5-fluorouracil (5-FU) from JW Pharmaceutical, and metformin from Sigma.

Figure 1. (Continued.)
E, Invasion and migration activity increased with CagA+ H. pylori infection, while no significant changes were noted after CagA– strain infection. F, The expression of Snail and Slug increased significantly upon infection with WT H. pylori. G, H. pylori infection and more advanced OLGIM staging were associated with stronger CDX1 expression (*, P < 0.05 vs. control, **, P < 0.01 vs. control, and *** P < 0.001 vs. control).
**H. pylori infection of HFE 145 cells**

Cells were seeded onto 6-well slide chambers or 6-cm-diameter petri dishes (Nunc). Cells were grown in medium with 2.5% FBS and without antibiotics for 20 hours at 37°C prior to *H. pylori* infection. Cells were washed once with sterile PBS, and then two *H. pylori* strains (60190 from ATCC, and G27, kindly provided by Prof. Nayoung Kim, Seoul National University, Korea) were added at a multiplicity of infection (MOI) of 100:1 for different time points, followed by washing with PBS six times to remove nonadherent bacteria. Isogenic mutants lacking CagA (60190ΔA) or CagE (G27AE) were used as described previously (19, 20).

**H. pylori infection of Rhesus macaque and gene expression analysis of gastric mucosa**

To compare the transcriptomic profiles of *H. pylori*-infected gastric mucosa and control, the expression levels of gastric mucosa were retrieved from a public transcriptome data (i.e., GSE8749; ref. 12). After log transformation of the expression levels, differential gene expression analysis was performed using GEO2R (https://www.ncbi.nlm.nih.gov/geo/geo2r/) and a heatmap was constructed using heatmap.2 function of gplots v3.0.1.1 in R.

**CDX1 expression vector and shRNA**

CDX1 was overexpressed using CDX1 lentiviral activation particles (sc-402522-LAC; Santa Cruz Biotechnology) on HFE 145 cell line. CDX1 was silenced via lentiviral transduction of particles (sc-402522-LAC; Santa Cruz Biotechnology) on HFE 145 expression vector and shRNA v3.0.1.1 in R.

**Luciferase reporter assay**

To confirm CagA induced CDX1 promoter activity, fragment of CDX1 promoter was amplified using two primers 5'-CTCGAGG-GATCCGGACTCCACAAA-3' and 5'-AAGCTTCAACCGCTCACCCTGAACTC-3', and then cloned into pGL3-basic luciferase vector using PCR-based technique. To measure binding site of CagA in CDX1 promoter, 1,000-bp fragment of CDX1 promoter was divided into Mut 500 and Mut 1000, and the same experiment of cloning was carried out (Mut 500 primers, 5'-CTCGAGG-GATCCGGACTCCACAAA-3' and 5'-AAGCTTCAACCGCTCACCCTGAACTC-3'; Mut 1000 primers, 5'-CTCGAGG-GATCCGGACTCCACAAA-3' and 5'-AAGCTTCAACCGCTCACCCTGAACTC-3'). Wild-type CagA isolated from *H. pylori* NCTC11637 strain was cloned into HA-tagged pSP65R vector (kindly provided by Prof. Yong Chan Lee, Yonsei University, Korea). HEK293T cell was seeded into 6-well plate at a density of 6 × 10^5 cells/well for 20 hours prior to cotransfection. Cells were cotransfected with 1.3 μg of CagA-CDNA psiP65R vector, 1 μg of CDX1 promoter luciferase plasmid, and 20 ng of pNL1.1 plasmid (Promega) using LTI (Mirus Bio LLC). After 2 days, cells was harvested, lysed, and analyzed for luciferase activity using NanoGlo Dual-Luciferase Reporter Assay System (Promega).

**Proliferation assay**

Cell proliferation was analyzed using the cell-permeable tetrazolium salt WST-1 (4-[3-[4-iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio]-1,3-benzene disulfonate) according to the manufacturer’s protocol (Roche Diagnostics GmbH). CDX1-overexpressing HFE 145, AGS, and MKN-28 cells were plated in triplicate in 96-well plates at a density of 5 × 10^3 cells/well. After 18 hours, serum-free medium and metformin were added to each well and incubated for 24 and 48 hours. After each time point, 10 μL WST-1 reagent was added to each well. The reaction proceeded for 4 hours at 37°C in 5% CO₂, and the optical density of each well was measured at 450 nm wavelength.

**Clonogenic assay**

CDX1-overexpressing HFE 145 cells suspended in DMEM with 10% FBS were seeded in 6-well culture plates at 1 × 10^3 cells/well. After 18 h, cells were then grown in fresh DMEM containing 0.1% FBS. The medium was replaced every 3 days. Two weeks later, colonies were stained with 0.05% crystal violet and counted. All clonogenic experiments were performed at least 3 times.

**In vitro migration and invasion assays**

Transfilter migration and invasion assays were performed in serum-free DMEM with 8.0-μm pore inserts in a 24-well Transwell apparatus (Corning Costar) following the manufacturer’s instructions and a previously published method (21, 22). After incubating for 18 hours, the membrane filter was collected, fixed, and stained, and invaded or migrated cells were counted in three randomly selected fields at × 100 magnification. All migration/invasion experiments were performed in triplicate.

**Western blot analysis**

Samples were prepared using RIPA buffer (Cell Signaling Technology), following transfer of electrophoretically separated proteins to a membrane. Expression of each protein was detected using an appropriate primary antibody. Antibodies against E-cadherin (#14472), ZO-1 (#13663), Slug (#2748), #3529, Oct4 (#2750), Nanog (#4893), cleaved Caspase-3 (#9661), and CD44 (#3578, #3570) were purchased from Cell Signaling Technology (1). Antibodies against Vimentin (sc-6260), CagA (sc-28368), c-Myc (sc-40), GAPDH (#5174), and β-actin (#4970) were purchased from Santa Cruz Biotechnology, and anti-Si was purchased from GenTex. Antibodies against Villin (ab130751), Sl (ab84977), MUC2 (ab212648), Ki-67 (ab16667), MMP-2 (ab92536) and MMP-9 (ab119906) were purchased from Abcam (Cambridge, UK), and anti-MUC5AC was purchased from LS Bio. An antibody against E-cadherin (BD610181) was purchased from BD Biosciences and anti-CDC44-PE (FAB6127P) from R&D Systems. Immunostaining was detected using an enhanced chemiluminescence system (Amer sham Biosciences) according to the manufacturer’s instructions.

**Flow cytometry**

For FACS, cells were dissociated using Accutase (BD561527, BD Biosciences) and resuspended in PBS containing 0.5% BSA. The cells were stained with FITC-conjugated CD44 (BD555478) or isotype control antibody (BD555742) from BD Biosciences on ice for 30 minutes. Cells were then washed with PBS and analyzed on a BD FACS Calibur (BD Biosciences) using Cell Quest software.
Soft-agar colony formation assay
Anchorage-independent growth was assayed by soft-agar colony formation assay. Briefly, each well of a 6-well culture dish was coated with 2 mL bottom agar mixture [Duchefa, 10% FBS, and 0.6% (w/v) agar]. After the bottom layer solidified, 2 mL top agar–medium mixture [DMEM, 10% FBS, and 0.3% (w/v) agar] containing 1 × 10^5 cells was added. Dishes were then incubated at 37°C in a 5% CO2 humidified atmosphere. Additional medium was overlaid onto the agar, and cells were then allowed to grow undisturbed for 2 weeks. Colonies with a diameter ≥100 μm were counted.

Figure 2.
CDX1 overexpression and suppression induce distinct phenotype changes related to cell proliferation, invasion, and stemness. A, CDX1-overexpressing HFE 145 cells showed increased expression of intestinal markers, such as villin, SI, and MUC2. B and C, CDX1 overexpression was associated with significantly increased clonogenic ability and elevated invasion and migration activity. D, Cells also displayed increased expression of EMT-associated proteins, such as vimentin, MMP-2, and MMP-9. E, Immunofluorescent staining confirmed CDX-overexpressing HFE 145 cells expressed low levels of E-cadherin and high levels of villin. F-I, CDX1 knockdown in AGS and MKN28 cells resulted in suppression of intestinal markers, clonogenic activity, expression of EMT-associated markers, and invasion/migration activity (**, P < 0.05 vs. control; ***, P < 0.01 vs. control, and ****, P < 0.001 vs. control).
counted over 5 fields per well for a total of 15 fields from triplicate experiments.

**Xenograft model**

To generate the xenograft model, 5 × 10⁶ untreated AGS cells or AGS cells pretreated with shCDX1 were suspended in 100 µL Hank balanced salt solution and injected subcutaneously into the flanks of athymic 6- to 8-week-old male BALB/c nu/nu mice. Vehicle (PBS) or cisplatin (2 mg/kg) was administered intraperitoneally once a week, and/or 5 mg metformin was given intraperitoneally daily. Tumor volume was estimated as volume = length × (width)²/0.52.

**Patient samples**

Forty patients diagnosed with gastritis or gastric neoplasms between May 2012 and December 2012 at the National Cancer Center (Korea) were enrolled in our study. During endoscopic examination, biopsies were performed to confirm the diagnoses, and 7 additional specimens were obtained from each patient to determine underlying histology and H. pylori infection status. Two specimens were taken from the antrum lesser curvature and corpus lesser curvature for evaluating intestinal metaplasia using the updated Sydney system (23) and staging intestinal metaplasia according to the Operative Link on Gastric Intestinal Metaplasia Assessment (OLGIM) system (24). H. pylori infection status was determined using a rapid urease test, Wright–Giemsa staining of the greater curvature of the stomach, or serologic testing; infection with H. pylori was confirmed if any of the above tests was positive. CDX1 expression was determined from samples of the corpus lesser curvature using Western blotting and densitometry.

**IHC and immunofluorescence**

Representative tissue samples were obtained from study participants and from murine xenografts. Formalin-fixed,
paraffin-embedded sections were processed in a previously described manner (22). To evaluate apoptosis, metastasis, and stemness, sections were incubated with respective antibodies against CD44, SOX2, and cleaved caspase-3 and analyzed as described previously (25).

Statistical analysis
Statistical Package for the Social Sciences version 21 (IBM SPSS, Inc.) was used for statistical analyses. All experiments were performed in triplicate (at minimum), and results were presented as means ± SDs. The Student t test was used to identify significant differences between experimental and control groups. P < 0.05 indicated statistical significance.

Study approval
Xenograft protocols were approved by the Institutional Animal Care and Use Committee of Memorial Sloan Kettering Cancer Center (New York, NY). Written informed consent was acquired from all human participants. The study was approved by the Institutional Review Board of National Cancer Center, Korea (IRB no. NCCNCS-12-595).

Results
H. pylori infection changes HFE 145 cell phenotype
Upon infection with H. pylori wild-type (WT) 60190, HFE 145 cells elongated compared with cells infected with CagA-deficient H. pylori 60190, which is commonly called as “hummingbird phenotype” (Supplementary Fig. S1B). Infection with WT H. pylori led to significantly increased numbers of Ki-67+ epithelial cells at 6 and 24 hours in immunofluorescence assays compared with cells infected with the CagE-deficient G27 [P < 0.001 and 0.043 (control vs. WT); P = 0.081 and 0.494 (control vs. mutant) at 6 and 24 hours, respectively] and CagA-deficient 60190 [P = 0.011 and 0.029 (control vs. WT); P = 0.089 and 0.499 (control vs. mutant) at 6 and 24 hours, respectively] strains (Supplementary Fig. S1C).

We evaluated the CDX1 expression status in various gastric cancer cells and found out that several gastric cancer cells had CDX1 expression in their native status (Fig. 1A). Expression of CDX1 in the HFE 145 cells increased significantly when infected with WT H. pylori, but not with the CagA-deficient mutant (P = 0.002 and 0.010 compared with control; Fig. 1B; Supplementary Fig. S1D). Also, the expression of proteins that are associated with stemness, such as SOX2 and Nanog increased as well (Fig. 1C; Supplementary Fig. S1E). Luciferase reporter assays confirmed that CDX1 induction is CagA-dependent. Deletion of 501 to 1,000 base pairs upstream of CDX1 exon did not suppress the luciferase activity, while deletion of 1 to 500 base pairs of the same lesion inhibited its activity (Fig. 1D). It implies that potential binding site of CagA associated factor is located at designated area. Increased luciferase activity with truncated mutation preserving 1 to 500 base pairs upstream CDX1 exon is probably due to increased binding affinity due to shortened promoter area.

HFE 145 cells infected with H. pylori WT 60190 showed significantly increased invasion and migration activity in transmembrane assays, while no significant difference was noted with CagA-deficient 60190 infection [P = 0.0378 and 0.040 (control vs. CagA'); 0.136 and 0.356 (control vs. CagA') for invasion and migration, respectively; Fig. 1E]. Moreover, epithelial cells infected with WT H. pylori 60190 showed higher levels of Snail and Slug on immunofluorescence assay, further indicating the cells’ increased invasiveness and migration [P = 0.002 (control vs. CagA') vs. 0.836 (control vs. CagA') for Snail and P < 0.001 (control vs. CagA') vs. 0.150 (control vs. CagA') for Slug; Fig. 1F]. These findings suggest that CDX1 is upregulated in epithelial cells exposed to CagA, a major virulence factor of H. pylori, and promotes their invasion and migration.
CDX1 expression in patient tissue is associated with intestinal metaplasia

Several patient samples were evaluated to investigate the possible relationship between CDX1 expression in gastric epithelial cells and intestinal metaplasia stage. Samples #5 and #7 were excluded from statistical analysis because they were collected from the same patient (#6). Tissue samples with confirmed *H. pylori* infection tended to have higher expression of CDX1 (\(P = 0.061\)). And those with more advanced IM (stage 2, 3, or 4) exhibited significantly greater expression of CDX1 (\(P = 0.039\); Fig. 1G). These differences in CDX1 expression status according to the *H. pylori* infection status and the severity of intestinal metaplasia concurs with and support the previous *in vitro* findings.

Figure 4.
Metformin suppresses effects of CDX1 in CDX1-expressing cells. A and B, Metformin significantly decreased the expression of CDX1 and intestinal markers villin, SI, and MUC2, restored expression of E-cadherin, and inhibited invasion and migration activity. C and D, Metformin inhibited spheroid formation in AGS and MKN28 cells, especially in CD44+ cells. (Continued on the following page.)
CDX1 promotes intestinal metaplasia marker expression, cell proliferation, and invasion/migration

We investigated the biological features of CDX1-overexpressing gastric epithelial cells generated through lentiviral-mediated transduction and gastric cancer cells natively expressing CDX1. Among CDX1-expressing gastric cancer cell lines, AGS and MKN-28 were used in this study. CDX1-overexpressing gastric epithelial cells showed increased expression of intestinal differentiation markers Villin, SI, and MUC2 (Fig. 2A). To determine the function of CDX1 in this context, we examined its effect on cell growth in clonogenic soft agar and transmembrane invasion and migration assays. CDX1-overexpressing cells yielded 14-fold more colonies than controls over 2 weeks after seeding the cells ($P < 0.001$; Fig. 2B) and exhibited significantly increased invasion and migration activity ($P = 0.007$ and $P = 0.021$, respectively; Fig. 2C). Western blotting revealed decreased E-cadherin expression and increased vimentin and MMP-9 expression (Fig. 2D), and immunofluorescence assays confirmed decreased E-cadherin and increased villin in CDX1-overexpressing HFE145 cells (Fig. 2E).

In AGS and MKN28 gastric cancer cells natively expressing CDX1, we observed significantly decreased expression of Villin, SI, and MUC2 after shRNA-mediated suppression of CDX1 (Fig. 2F). AGS and MKN28 cells treated with shCDX1 did not significantly inhibit cell growth compared with controls at 1, 3, and 5 days ($P = 0.0064$, 0.454, and 0.693 for AGS cells; $P = 0.069$, 0.550, and 0.645 for MKN28 cells, respectively; Supplementary Fig. S2A). However, among diabetic patients, metformin users had relatively lower CDX1 expression compared with metformin nonusers as determined by densitometry measures of CDX1 normalized to actin (‘, $P < 0.05$ vs. control, **, $P < 0.01$ vs. control, and ***, $P < 0.001$ vs. control).
CDX1 suppression markedly decreased cell survival, resulting in inhibited colony formation activity ($P < 0.001$ and $P = 0.001$, respectively; Fig. 2G). Expression of EMT-associated proteins, such as SOX2, Oct4, and c-Myc, was also suppressed by suppression of CDX1 (Fig. 2H). Finally, CDX1 knockdown led to decreased invasion and migration by 81.7% and 84.4% in AGS cells and by 75.5% and 76.9% in MKN28 cells ($P = 0.001$ and $P = 0.001$ for AGC cells; $P = 0.001$ and $P = 0.004$ for MKN28 cells, respectively; Fig. 2I; Supplementary Fig. S2B).

**CDX1 overexpression induces a cancer stem cell–like phenotype in gastric epithelial cells**

CDX4$^+$ cells comprised approximately 6.9% of CDX1-overexpressing HFE 145 spheroids compared with 4.5% in WT HFE 145 spheroids ($P = 0.025$; Fig. 3A; Supplementary Fig. S3A). CD44, a potential marker for cell stemness in gastric cancer, was not significantly expressed when HFE 145 cells and gastric cancer cells were cultured in monolayers; however, its expression was prominent when these cells were cultured in spheroids (Supplementary Fig. S3B and S3C). And CDX1-overexpressing HFE 145 spheroids exhibited higher expression of CD44 and other stemness-associated proteins, such as SOX2, Oct4, and c-Myc, compared with WT HFE 145 spheroids (Fig. 3B). The number of spheroids was also significantly higher in CDX1-overexpressing HFE 145 cells compared with vector-treated cells ($P = 0.001$; Fig. 3C). These results imply that CDX1 endows a cancer stem cell–like phenotype in normal gastric epithelial cells by upregulating expression of self-renewal proteins.

Expression of CD44, SOX2, Oct4, and Nanog decreased after CDX1 suppression in AGS and MKN28 cells (Fig. 3D and E). We also observed a substantial decrease in the number of spheroids formed by shCDX1-treated AGS and MKN28 cells ($P = 0.001$ and $P = 0.004$, respectively; Fig. 3F), further indicating the potential association between CDX1 expression and a stem cell–like phenotype. This association was further validated through evaluation of tissues from patients with gastric cancer. Tissues with high CDX1 expression showed stronger expression of CD44 and SOX2 in both IHC and immunofluorescence assays (Fig. 3G).

**Metformin attenuates expression of intestinal markers and the invasive phenotype of CDX1-overexpressing cells**

When CDX1-overexpressed HFE 145 cells were treated with metformin, we observed reduced expression of CDX1, Villin, Sl, and MUC2 and restoration of E-cadherin expression (Fig. 4A). Invasion and migration were also inhibited with metformin treatment in these cells ($P < 0.001$ and $P = 0.027$, respectively; Fig. 4B). Intestinal markers, such as Sl and MUC2, and invasion and migration activity were suppressed when AGS and MKN28 cells were exposed to metformin ($P < 0.001$ and $P = 0.002$, respectively; Supplementary Fig. S4A and S4B). Markers associated with EMT, including Vimentin and Snail, were significantly reduced upon exposure to metformin in gastric cancer cells (Supplementary Fig. S4C). These findings suggest that metformin reverses or suppresses the phenotypic changes that arose in association with CDX1 expression.

Spheroid-forming activity in AGS and MKN28 cells was significantly inhibited at 48 and 96 hours after treatment with metformin ($P = 0.009$ and $0.003$ for AGS; $P = 0.027$ and $0.002$ for MKN28, respectively; Fig. 4C) and this change was more prominent in CD44$^+$ spheroids ($P < 0.001$ and $P = 0.001$ for AGS and MKN28, respectively; Fig. 4D). Expression of CD44, SOX2, Oct4, Nanog, Vimentin, MMP-2, and MMP-9 in CD44$^+$ AGS and MKN28 spheroids was also suppressed upon exposure to metformin (Fig. 4E and F). Invasion and migration were more prominent in AGS and MKN28 spheroids than corresponding monolayer cells ($P = 0.006$ and $0.003$ for AGS; $P = 0.002$ and $0.002$ for MKN28, respectively; Supplementary Fig. S4D) but were significantly inhibited in the presence of metformin ($P = 0.001$ and $0.001$ for AGS; $P < 0.001$ and $0.002$ for MKN28, respectively; Fig. 4G). This close association between CD44 and CDX1 expression was clearly visualized in immunofluorescence assays (Fig. 4H). Above findings imply that metformin also inhibits stem cell–like phenotypes that were found in CDX1-expressing cells.

In analyses of patient tissues with diabetes, metformin users exhibited relatively lower CDX1 expression as determined by densitometry measures of CDX1 normalized to actin [0.727, 0.965, 1.237, and 0.318 vs. 1.345 and 1.131 (mean = 0.812 vs. 1.238); Fig. 4I].

**CDX1 suppression and metformin treatment exerted similar effects in an AGS xenograft mouse model**

AGS cells treated with cisplatin and fluorouracil (5-FU) showed marked expression of cleaved caspase-3 and a subsequent decrease in the number of viable cells, implying increased apoptotic activity upon exposure to chemotherapeutic agents. When shCDX1 was coadministered with 5-FU or cisplatin, more prominent changes were noted. While we observed a 20.1% or 26.5% reduction in proliferation with exposure to 5-FU or cisplatin in AGS spheroids ($P = 0.003$ or $P = 0.001$, respectively), we noted a significant 48.6% or 53.0% reduction when shCDX1 was used with 5-FU or cisplatin ($P < 0.001$ or $< 0.001$, respectively; Fig. 5A).

The effects of chemotherapeutic agents varied according to the culture status of AGS cells. For example, spheroids were more resistant to 5-FU and cisplatin and showed only a 18.5% or 17.4% reduction in proliferation ($P = 0.012$ or 0.021, respectively) compared with monolayer cells, which showed 48.5% or 56.5% reduction ($P < 0.001$ or $< 0.001$, respectively; Supplementary Fig. S5A). The suppression profiles of AGS cells treated with shCDX1 implies that knockdown of CDX1 may abolish the stem cell–like features, so called "chemoresistance" of CDX1-expressing AGS spheroids.

To demonstrate the in vivo effect of CDX1 knockdown, we used an AGS xenograft mouse model. When shCDX1 was applied to tumor cells before inoculation into mice, tumor growth was inhibited by 38.9% ($P = 0.027$ when compared with control), similar to 42.7% ($P = 0.020$ when compared with control) inhibition seen in mice treated with cisplatin at day 15. Mice inoculated with shCDX1-treated AGS cells and subsequently treated with cisplatin exhibited robust tumor volume reduction, 75.0% at day 15 compared with controls ($P < 0.001$; Fig. 5B). Mice did not show significant weight loss during the observed period, suggesting no obvious adverse events associated with the use of either shCDX1 or cisplatin ($P = 0.977, 0.987, 0.101, and 0.191$ for control, cisplatin, shCDX1, and cisplatin with shCDX1, respectively; Supplementary Fig. S5B). Tumor tissues were harvested from the mice to evaluate differences among the treatment groups. Significantly increased cleaved caspase-3 was noted in immunofluorescence assays in tissues treated with cisplatin or...
shCDX1 as well as trend toward decreased CD44 and MMP9 expression, and more notable changes were observed when cisplatin and shCDX1 were used together (Fig. 5C and D).

On the basis of previous experiments that showed the suppressive effect of metformin on CDX1-expressing cells, we repeated these assays in the xenograft model using metformin instead of shCDX1. When metformin was administered to the mice, tumor growth was inhibited by 53.7% ($P = 0.013$ when compared with control), compared with a 40.9% ($P = 0.013$ when compared with control) reduction after cisplatin treatment. Mice treated with both metformin and cisplatin showed pronounced effects, such as 77.0% reduction in tumor volume at day 15 ($P = 0.001$; Fig. 6A). Evaluation of tissues harvested from the xenografts revealed a decreased Ki-67$^+$ cells, suppressed expression of CDX1, and increased caspase-3 activity with the use of cisplatin or metformin, which was magnified with the use of both agents together (Fig. 6B and C). Obvious morphologic differences were noted upon visual evaluation of the tissues.

Figure 5.
CDX1 suppression inhibits tumor growth in an AGS xenograft model. A, Cisplatin (5 μmol/L) and 5-FU (5 mmol/L) exerted more pronounced effects when AGS cells were pretreated with shCDX1. B, In an AGS xenograft model, both shCDX1 and cisplatin showed significant inhibitory effects on tumor growth, especially when both agents were used together. C and D, Evaluation of harvested tissues exhibited that cisplatin with CDX1 suppression substantially increased apoptosis and decreased numbers of CD44$^+$ and MMP-9–expressing cells ($*, P < 0.05$ vs. control, $**, P < 0.01$ vs. control, and $***, P < 0.001$ vs. control).
flank tumor masses, highlighting the robust impact of cisplatin and metformin cotreatment (Fig. 6D). Flank tumors from control mice showed high infiltration toward adjacent tissues compared with xenograft tumors exposed to cisplatin and metformin, which showed clear demarcation between tumor and normal tissues (Fig. 6E). Immunofluorescent evaluation of tumor borders confirmed our microscopic findings and showed significantly decreased CD44 and MMP-9 in mice treated with both agents (Fig. 6F).

Discussion

We found that aberrant expression of CDX1, which can be triggered by CagA secreted from *H. pylori*, promotes cell
proliferation, invasion/migration, intestinalization of gastric epithelial cells, and induction of a stem cell-like phenotype that leads to cancer development and resistance to common gastric cancer chemotherapeutic agents. These findings suggest a possible mechanism underlying the relationship between CDX1 expression and development of precancerous and neoplastic lesions observed in other studies (13–16). We also showed that knockdown of CDX1 reverses these changes and enhances gastric cancer sensitivity to chemotherapeutic agents.

Our results, together with prior findings, indicated that CDX1 is an oncogene that induces intestinal transdifferentiation, EMT, and a stem cell–like phenotype in gastric epithelial cells, supporting intestinal-type gastric adenocarcinoma formation. CDX1 expression may be induced by H. pylori infection, which leads to chronic inflammation, followed by increased apoptosis of gastric epithelial cells that results in atrophy, alteration of differentiation status, metaplasia, and appearance of enterocyte-like cells expressing intestinal markers (26). CDX1 upregulates transcription factors, such as SALL4, KLF5 (27), and PPARγ (28), that facilitate the transdifferentiation of gastric epithelial cells to intestinal epithelial cells (21, 27). CDX1’s induction of PPARγ may help explain the finding in a recent study of 668 patients with gastric cancer that PPARγ expression predicts good prognosis in patients with intestinal-type, but not diffuse-type gastric cancer (21). As CDX1 has also been reported to be expressed at higher than normal levels in gastric and esophageal tissue displaying intestinal metaplasia (13–15), the functions revealed herein may help provide a mechanistic basis for understanding the increased risk of gastric cancer in the presence of atrophic gastritis and intestinal metaplasia (24, 29–32).

The observations that CDX1 knockdown reversed the invasive phenotype of CDX1-expressing cells, decreased expression of intestinal markers and EMT-associated proteins, and restored expression of normal epithelial markers support the idea that intestinal metaplasia may be at least partially reversible. The reversibility of intestinal metaplasia with elimination of H. pylori is still controversial, although a recent study found that H. pylori eradication may partially reverse precancerous lesions (33). Our results support the use of agents that may inhibit the phenotypic changes that arise in association with CDX1 expression, such as metformin after development of atrophy or intestinal metaplasia to prevent the development of gastric cancer.

Our findings suggest that the antidiabetic drug metformin can exert similar effects in vitro and in vivo as CDX1 suppression: reversal of the cancer stem cell–like phenotype, inhibition of tumorigenesis and tumor growth, and improvement in the efficacy of greater sensitivity to certain chemotherapeutic agents. However, we were not able to show the mechanism underlying these changes. We chose to investigate metformin’s efficacy based on evidence that it decreased risk of certain cancers (34–36), including gastric cancer (37, 38). Several mechanistic hypotheses have been proposed to explain this phenomenon, such as metformin’s suppression of cell-cycle–associated proteins (39), inhibition of the mTOR pathway through activation of AMPK (40), and suppression of insulin secretion and IGF-1 signaling (41). Thus, CDX1 might be another incidental target of metformin, or metformin’s inhibition of other pathways may lead to reduced CDX1 expression. Alternatively, recent studies have shown that certain cancer cell population with tumor initiating potential replies more on oxidative phosphorylation rather than glycolysis for their energy generation, unlike general cancer cell population (42–44). Because metformin is a potent inhibitor for oxidative phosphorylation, it is possible that it has suppressed the activity of these oxidative phosphorylation dependent cancer stem cells, which happened to be a major feature of CDX1-expressing cells. This could explain the reductions in CDX1 expression, we observed following metformin treatment of xenografted mice. In addition, a recent study reported that metformin may inhibit H. pylori growth in vitro and in vivo (45). Although further studies are needed to elucidate the underlying mechanism, we showed that metformin may reduce chronic gastric inflammation–associated changes and carcinogenesis.

We found that CDX1–overexpressing cells cultured as spheroids included a larger population of cells expressing cancer stem cell marker CD44 (22, 46) compared with non-CDX1–overexpressing spheroids, and displayed stronger expression of other stemness markers. In addition, knockdown of CDX1 decreased expression of stemness markers, suppressed spheroid formation, and reduced invasion/migration potential to a greater degree among CD44-positive cells. These findings suggest that CDX1 is associated with acquisition of stemness in gastric epithelial cells, and that this change supports tumorigenesis. We also showed that targeting CDX1-expressing cells may suppress the development of cancer and improve response to chemotherapy. Adverse effects associated with suppression of CDX1 and targeting cancer stem cells may be of concern, but we showed that the systemic toxicity of CDX1 suppression achieved by metformin may not be significant in vivo. Further investigation is needed to explore the utility and safety of this strategy for chemoprevention and treatment of gastric cancer.

In conclusion, we showed that the aberrant expression of CDX1, which is often induced by the virulence factor CagA of H. pylori, contributes to gastric cancer tumorigenesis and progression. We also demonstrated that the cumulative effects of H. pylori infection, including increased proliferation, invasiveness, and stemness of gastric epithelial cells, can be partially reversed by targeted suppression of CDX1. Additional investigations are warranted to determine whether CDX1 is an ideal candidate for targeted chemotherapeutic treatment of gastric cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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