Overexpression of Cdx2 inhibits progression of gastric cancer *in vitro*

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Abstract. The caudal-type homeobox gene Cdx2 encodes a transcription factor which in adult mammals is expressed in the cells of the intestinal epithelium and is thought to play an important role in their proliferation and differentiation. Cdx2 mediates apoptosis and suppresses tumorigenesis in many tissue types, but there are few data available on Cdx2 expression and its relationship to tumor kinetics in gastric cancer. To gain better insight into the involvement of Cdx2 in the biological characteristics of gastric cancer, we investigated the effect of Cdx2 overexpression on the progression of gastric carcinoma cells. A gastric cancer cell line stably overexpressing Cdx2 (MGC-803/Cdx2) was established. The influence of Cdx2 overexpression on *in vitro* cell growth was assessed by measuring cell survival, colony formation and cell cycle progression. The results clearly showed that overexpression of Cdx2 significantly inhibited cell growth and proliferation, blocking entry into the S phase of the cell cycle. MGC-803/Cdx2 cells also had a higher apoptotic rate than control cells. In addition, Cdx2 reduced the motility and invasion of gastric cancer cells. In summary, Cdx2 overexpression can effectively inhibit gastric cancer progression and may be used as a potent therapy.

Introduction

Homeobox genes encode transcription factors that play crucial roles in early developmental processes including patterning and cell identity. Some are expressed in adulthood and alterations are linked to malformations, metabolic diseases, and cancers (1). Cdx2 gene, a member of the caudal-related homeobox gene family, homologous to the *Drosophila* gene caudal, has an essential role during early development. The Cdx2 gene is an intestinal transcription factor that may be involved in the regulation of the proliferation and differentiation of intestinal epithelial cells (2,3). Cdx2 protein is predominantly expressed in the intestine and colon, but not in the normal epithelium of the esophagus and stomach through adulthood in humans and mice.

Initially, homeobox genes was suggested to behave as proto-oncogenes because substantial evidence indicates that their alterations participate in tumor genesis (4). For example, overexpressing Cdx1 in the intestinal cell line has been reported to increase proliferation and differentiation but also transformational and tumorigenic activity. Meanwhile, Cdx2 induces intestinal metaplasia of stomach, esophagus and other issues and Cdx2-induced intestinal metaplasia itself causes carcinoma (5,6). However, as a nuclear transcription factor, Cdx2 has also been proved to be a tumor suppressor. A number of studies performed on several cancer cell lines have provided evidence that Cdx2 inhibits cell growth, stimulates overall cell differentiation and also promotes cell apoptosis (7,8). These results indicate that Cdx2 opposes the malignant behaviour of cancer cells. Apart from the effects reported on cell lines, a link has been proposed between colon cancers and Cdx2 status as human colorectal cancers and chemically induced colon tumors in the rat show reduced Cdx2 levels in relation to tumor grade (9,10).

Gastric carcinoma remains the second most common cause of cancer deaths on a worldwide basis. In China, there are 400,000 new cases of gastric cancer and 300,000 deaths annually, making gastric cancer the third most common type of cancer in China (11). Dysregulation of gene expression is integral to the neoplastic process, and there is now compelling evidence implicating upregulation or downregulation of multiple genes in the development and progression of gastric cancer. However, the clinical significance and implication of Cdx2 expression in human gastric carcinoma are unclear.

We conducted this study to investigate the involvement of Cdx2 activity in the development and progression of gastric cancer. In this study, we constructed an eukaryotic expression vector with Cdx2 gene to determine the effects of Cdx2 overexpression on biological characteristics of gastric cancer in an *in vitro* cell model for gastric cancer. The long-term goals of our project are to exploit the potential use of Cdx2 as a marker for clinical diagnosis and prognosis of

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Table I. The base sequence of primers for RT-PCR.

Gene	Primer	Base sequence	PCR product (bp)
Cdx2	Forward	5'-CCAAGCTTGCCACCATGTACGTGAGC-3'	961
	Reverse	5'-CGGAATTCCTGGGTGACGGTGGGGTT-3'	
GAPDH	Forward	5'-AACGGATTTGGTCGTATTG-3'	210
	Reverse	5'-CTGGAAGATGGTGATGGG-3	

gastric carcinomas and as a target for the development of therapeutic approaches to treat this disease.

Materials and methods

Cell culture. The poorly differentiated human gastric adenocarcinoma cell line, MGC-803, was purchased from Cell Bank, Chinese Academy of Science, Shanghai, China, and cultured in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Gaithersburg, MD, USA). All media were supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were cultured in an incubator at 5% CO_2 in air at 37˚C, with medium changes every 3 days.

Construction and transfection of the Cdx2 plasmid expression vector. Plasmid pCMV-HA, purchased from Shanghai Cancer Institute, China, was used to construct the Cdx2 expression vector. The methods of pCMV-Cdx2-HA and transfection of MGC-803 gastric cancer cells with pCMV-HA or pCMV-Cdx2-HA have been previously described (12). Briefly, a Cdx2 cDNA PCR product and pCMV-HA were digested with *Eco*RI. The digested PCR product was electrophoresed through and isolated from an agarose gel. After purification, it was ligated into the cut vector to form pCMV-Cdx2-HA. After the ligation, the plasmid was transformed into *Escherichia coli* TOP10 cells, and then planted on solid LB medium. Ampicillin-resistant colonies were cultured at 37˚C overnight in a rocking bed. The recombinant plasmid was prepared, and the sequences were verified by electrophoresis of the digested product. MGC-803 cells ($1x10^5$) were inoculated into a 6-well plate and transfected with pCMV-Cdx2-HA or pCMV-HA recombination plasmids when the confluence was 90%. Forty-eight hours after transfection, MGC-803 cells were diluted to 1:10 for passage, and cultured for at least 2 weeks in medium with G418.

RT-PCR analysis. Total cellular RNA was extracted from cells using TRIzol Reagent (Invitrogen) according to the manufacturer's instructions. RNA concentration was determined by measuring UV absorption. RNA was reverse transcribed to single-strand cDNA using a Revertaid First Strand cDNA Synthesis Kit (Fermentas International Inc., Canada). Table I shows the PCR primer sequences used in this study. The reaction contained 2 μg RNA, 1 μl random primer and DEPC-treated water added to the sample to a volume of 12 μl, incubated at 70˚C for 10 min and put on ice. Afterwards 4 μl of reaction buffer, 2 μl of dNTPs and 1 μl of RNase inhibitor were added. Samples were incubated at 25˚C for 5 min, and added 1 μl of M-MuLV reverse transcriptase was introduced. After incubation at 25˚C for 10 min, the samples were finally heated at 48˚C for 60 min. The reactions stopped by heating at 70˚C for 10 min, and the samples chilled on ice. The amplification for Cdx2 was done under the following conditions: 25 μl reaction mixture contained 2.5 μl of PCR buffer, 2 μl of dNTP, 2.5 units of Takara LA Taq polymerase (Takara, Japan), 1 μl of each primer, and 100 ng of cDNA at 94˚C for 5 min; then 94˚C for 45 sec, 56˚C for 30 sec, 72˚C for 1 min for 35 cycles, and finally at 72˚C for 10 min for balance. After PCR reaction, 2 μl of the amplified product was electrophoresed in 2% agarose gelatin gels buffered withc.

Western blot analysis. Cell lysates were prepared in a buffer containing 100 mM NaCl, 10 mM Tris-Cl (pH 7.6), 1 mM EDTA (pH 8.0), 1 μg/ml aprotinin, 100 μg/ml PMSF, and 1% (v/v) NP40. After protein quantification using the Lowry assay, equal amounts of protein were separated by SDS-PAGE and blotted onto nitrocellulose membranes by the semi-dry blotting method using a 3-buffer system. The membrane was blocked with 5% BSA in PBST (PBS, pH 7.5, containing 0.1% Tween-20) and incubated with a 1:1500 dilution of primary antibody (anti-Cdx2) (Pierce Biotechnology Inc., Rockford, IL, USA) overnight at 4˚C. The membrane was washed with PBST and incubated with a peroxidase-conjugated secondary antibody (1:2500) (Santa Cruz Biotechnology, Inc., CA, USA) for 1 h. Specific antibody binding was detected using a chemiluminescence detection system (Pierce) according to the manufacturer's recommendations. After the film had been developed, the membrane was stripped and reprobed with antibody against ß-actin (1:1000) (Santa Cruz Biotechnology Inc.) to confirm equal sample loading.

Cell proliferation and survival assay. The viability and proliferation of cells were determined by an MTS assay using a CellTiter 96 AQueous assay system (Promega, Madison, WI, USA), according to the manufacturer's instructions. Briefly, the cells were plated at $1x10^4$ cells/well in 96-well plates and cultured for 7 days. MTS mixed with medium without serum was added to the cell cultures after removal of the old medium. The plates were incubated at 37˚C for 2 h, and the absorbance (A) at 490 nm was determined using a 96-well Opsys MR™ microplate reader (ThermoLabsystems, Chantilly, VA, USA) and Revelation™ QuickLink Software. The blank control wells with medium only were set as zero absorbance controls. The percentage of cell survival was calculated using the background-corrected absorbance: %

proliferation rate = 100xA of experimental well/A of untreated control well. All experiments were performed at least three times.

Colony formation assay. Cell suspensions from each group were diluted in DMEM supplemented with 10% FBS, and immediately replated in 6-well plates at 20 cells/cm^2. The plates were incubated until the cells in the control wells had formed sufficiently large colonies. The colonies were fixed with 6% glutaraldehyde and stained with 0.5% crystal violet. The plates were photographed and their digital images were manually analyzed to determine the colony number.

Cell cycle analysis by flow cytometry. For cell cycle analysis, MGC-803 cells (1x10^6) were washed twice with ice-cold PBS, treated with trypsin, and fixed in cold 70% ethanol at 4˚C for 30 min. The cell pellet was incubated in a solution containing 50 ng/ml propidium iodide, 0.2 mg/ml RNase, and 0.1% Triton X-100 at room temperature for 30 min. The cells were analyzed by flow cytometry using an EPICS XL-MCL FACscan (Becton-Dickinson, Mountain View, CA, USA). The data were analyzed with the MultiCycle Software for Windows (Phoenix Flow Systems, San Diego, CA, USA).

Apoptosis assay by flow cytometry. Apoptotic cells were identified by the Annexin V-FITC Apoptosis Detection Kit (Jingmei Biotech Co., Shenzhen, China) and an EPICS XL-MCL flow cytometer (Becton-Dickinson) according to the manufacturer's instructions. Briefly, 1x10^6 cells were stained with Annexin V-FITC for 30 min at 4˚C in the dark and propidium iodide for 10 min before flow cytometric analyses.

Wound healing assay. The cells were cultured to confluence on 6-well plates. A central linear wound was made with a 200 μl sterile pipet tip. Media were changed gently to remove any floating cells. Phase micrographs of the wound cultures were taken at 0, 24, 48 and 72 h. The photographs were analyzed by measuring the distance from the wound edge of the cell sheet to the original wound site. Migration activity was calculated as the mean distance between edges of 3 points in 12 fields/well. Healing rate = (mean original distance - mean distance at a time point)/mean original distance x100%. Each test group was assayed in triplicate.

Cell invasion assay. Cell invasion was assayed using Transwell chambers (6.5 mm; Corning, New York, USA) with 50 μl serum-free DMEM containing 1 μg/μl Matrigel (Dept. of Biology, Beijing University, China) in the upper chamber. The lower chamber was filled with 50 μl DMEM containing 0.1 μg/μl fibronectin (Beijing University). Cells (1x10^5) were resuspended in 100 μl DMEM with 1% fetal calf serum and plated into the upper chamber. The lower chamber was filled with 600 μl DMEM containing 0.1% ABS. After incubation at 5% CO_2 in air at 37˚C for 24 h, the number of cells on the undersurface of the polycarbonate membranes that were stained with Giemsa was visually scored in 5 random fields at x400 magnification in a light microscope.

Statistical analysis. Data are expressed as mean ± standard error of the mean (SEM) analyzed by SPSS 13.0 (SPSS Inc., Chicago, IL, USA) and Origin 7.5 software programs (OriginLab Co., Northampton, MA, USA). Student's t-test was used to measure statistical significance between two treatment groups. Multiple comparisons were performed with a one-way analysis of variance (ANOVA). Data were considered significant at $P<0.05$.

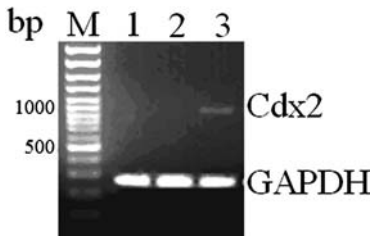

Figure 1. The expression of Cdx2 mRNA in gastric cancer MGC-803/Cdx2 cells was higher than that in MGC-803/EV or MGC-803 cells. Lane M, 1500 bp marker; lane 1, MGC-803 cells; lane 2, MGC-803/EV cells; lane 3, MGC-803/Cdx2 cells.

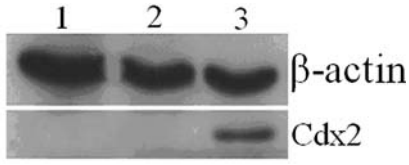

Figure 2. Western blot analysis of Cdx2 protein levels in human gastric carcinoma cells. ß-actin was utilized as a control to verify equal protein loading and transfer. Lane 1, MGC-803 cells; lane 2, MGC-803/EV cells; lane 3, MGC-803/Cdx2 cells.

Results

Construction and transfection with the pCMV-Cdx2-HA recombinant vector. To examine the effect of Cdx2 on gastric tumor cell progression *in vitro*, the plasmid pCMV-HA was used to construct the Cdx2 expression vector, pCMV-Cdx2-HA. The MGC-803 cell line was engineered to stably express increased levels of Cdx2 protein, and the engineered cell line is referred to as MGC-803/Cdx2. A control cell line was transfected with the empty vector and referred to as MGC-803/EV. mRNA and protein levels of Cdx2 in MGC-803 cells were validated by reverse transcription-polymerase chain reaction (RT-PCR) with primers specific to Cdx2 and Western blotting with antibody against Cdx2, respectively. An obvious 961 bp band was observed following gel electrophoresis of RT-PCR products amplified from MGC-803/Cdx2 cellular RNA, whereas no 961 bp band was observed following RT-PCR of MGC-803 or MGC-803/EV cellular RNA (Fig. 1). In addition, markedly higher levels of Cdx2 protein were expressed in MGC-803/Cdx2 cells. In contrast, no Cdx2 expression was observed in MGC-803 or MGC-803/EV cells (Fig. 2). The data suggest that the pCMV-Cdx2-HA recombinant vector was successfully constructed and Cdx2 was stably overexpressed in MGC-803/Cdx2 cells *in vitro*.

Cdx2 overexpression inhibits cell growth and proliferation in gastric cancer MGC-803 cells. We then determined the *in vitro* survival rates of MGC-803/Cdx2 cells. MGC-803/Cdx2 cells exhibited significantly reduced cell survival, as assessed by

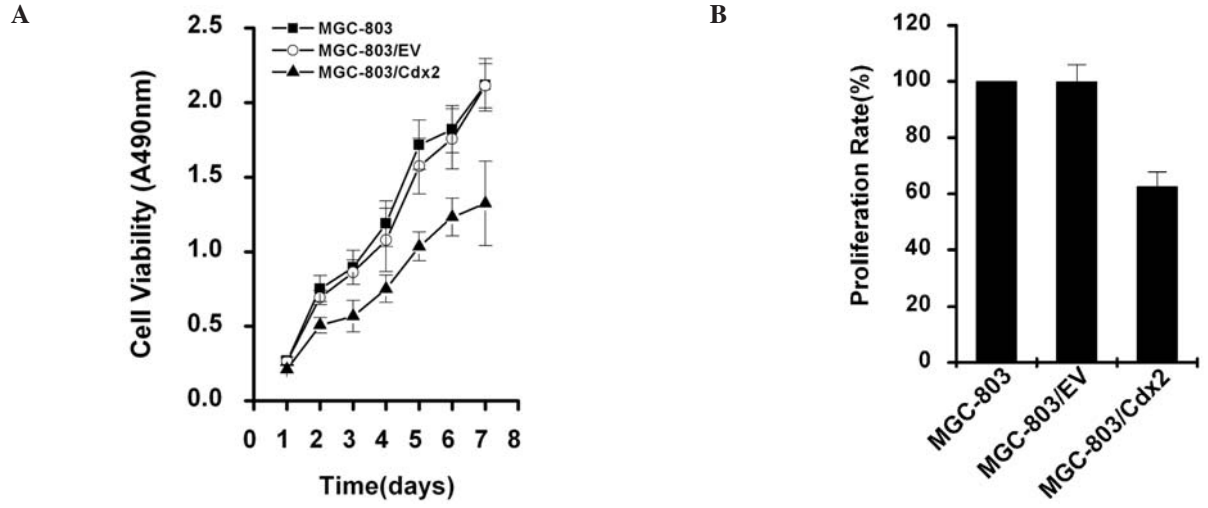

Figure 3. Cell survival was assessed by MTS assay. The results represent the means of at least three independent experiments. (A) The cell viability was observed after MTS treatment in MGC-803/Cdx2, MGC-803/EV and MGC-803 cells at day 1-7. (B) The mean cell proliferation rate of MGC-803/Cdx2 cells for 7 days after MTS treatment was significantly lower compared with that of MGC-803/EV or MGC-803 cells.

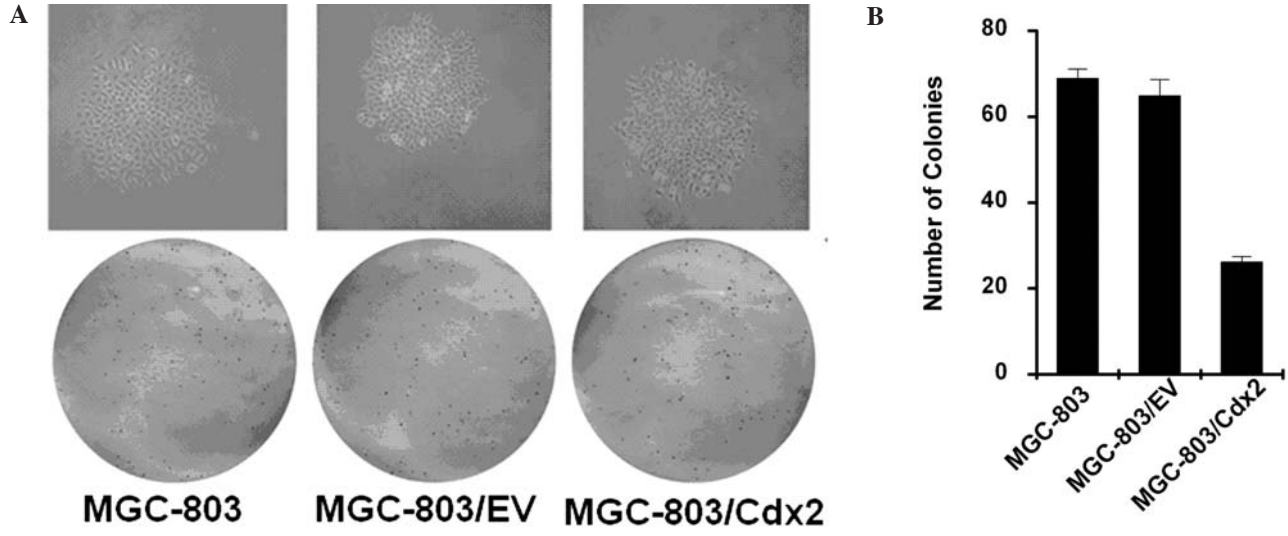

Figure 4. Results of colony formation assay. (A) MGC-803, MGC-803/EV and MGC-803/Cdx2 cells were plated in 6-well plates. The surviving fraction of cells (visible colonies) was stained with gentian violet, photographed and counted manually. (B) MGC-803/Cdx2 cells exhibited fewer colonies than MGC-803/EV cells or MGC-803 cells.

the MTS assay (Fig. 3). MGC-803/EV and MGC-803 cells had about 1.6-fold higher mean proliferation rates than MGC-803/Cdx2 cells (P<0.05). We also observed that MGC-803/Cdx2 cells grew slower than MGC-803 and MGC-803/EV cells, consistent with the elevated levels of Cdx2 in MGC-803/Cdx2 cells and indicative of a suppressive effect of Cdx2 on MGC-803 cell growth and survival.

To confirm the inhibitory effect of Cdx2 on the growth of MGC-803 cells, colony formation was assayed to check the capability of the cell lines to grow in an anchorage-independent environment (soft agarose). The three cell lines were able to form colonies in soft agarose, but the number and size of the colonies were not consistent across the cell lines (Fig. 4A). The number of colonies formed by MGC-803/Cdx2 cells after 3 weeks of culture was 26.1±1.3, which represents a 59.7 and 62.1% decrease, respectively, when compared with the MGC-803/EV and MGC-803 cells (P<0.05) (Fig. 4B). Together, the data suggest that Cdx2 inhibits cell growth and proliferation in the gastric cancer cell system.

Effect of Cdx2 overexpression on cell cycle control in gastric cancer MGC-803 cells. Flow cytometry was used to determine whether the inhibitory effect of Cdx2 on MGC-803 cell proliferation was mediated, at least in part, by cell cycle progression. The following cell cycle profile for MGC-803/Cdx2 cells was seen: 47.1% were in G0/G1 phase and 35.8% were in S phase, with a 32.3 and 38.9% increase in G0/G1 phase cell population and a 26.5 and 31.5% decrease in S phase cell population compared to MGC-803/EV and MGC-803 cells, respectively (P<0.05) (Table II and Fig. 5). The data indicate that cell growth inhibition by Cdx2 is associated with significant cell cycle arrest at the G0/G1 phase and

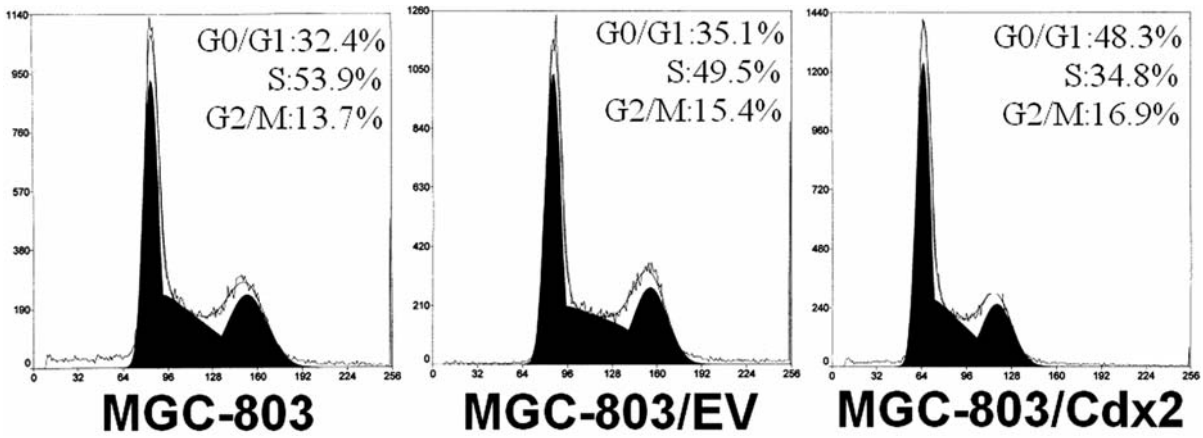

Figure 5. Cell cycle analysis by flow cytometry in MGC-803, MGC-803/EV and MGC-803/Cdx2 cells. The cell cycle distribution of the propidium iodide-labeled cells was analyzed by flow cytometry.

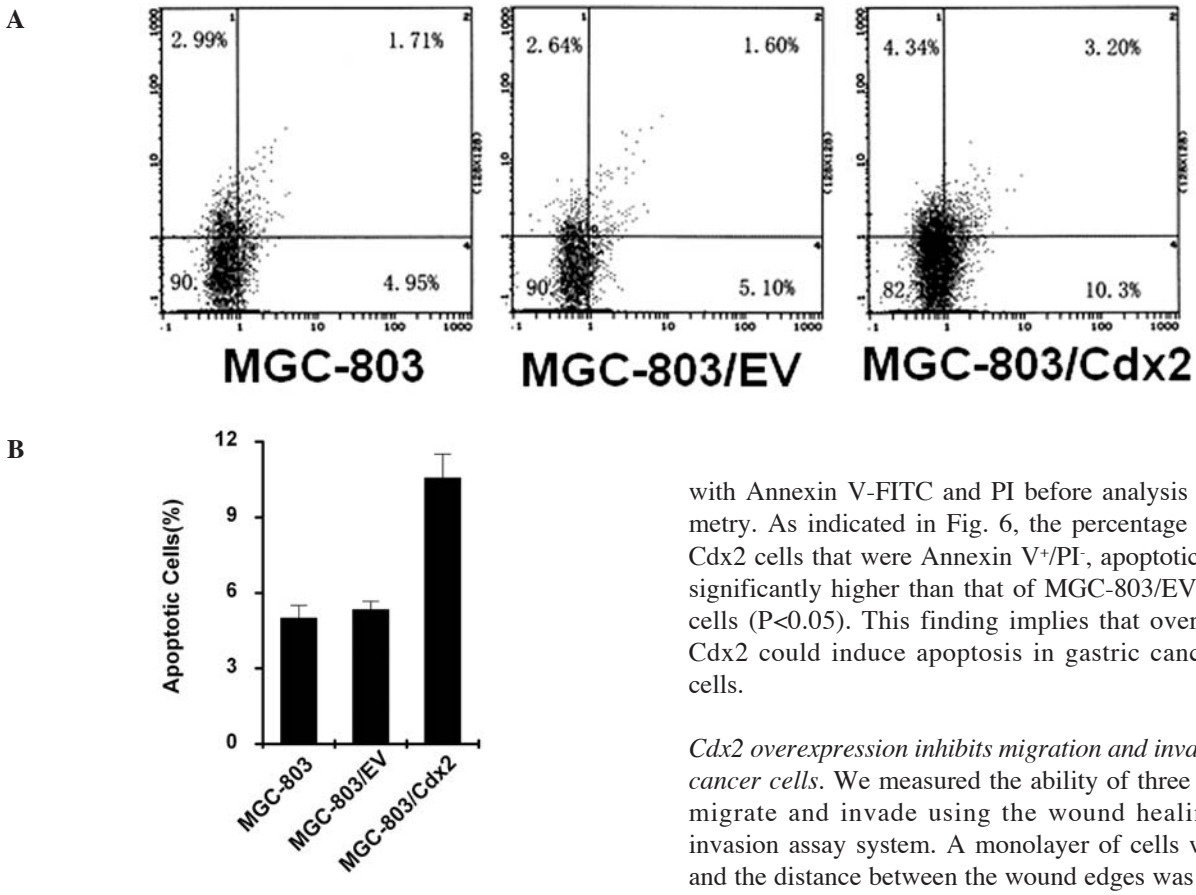

Figure 6. Percentages of apoptotic cells analyzed by flow cytometry. (A) Numbers in the quadrants reflect the percentage of MGC-803, MGC-803/EV and MGC-803/Cdx2 cells. Representative flow cytometry results were shown for the three groups. (B) The mean apoptotic rate in MGC-803/Cdx2 cells was significantly higher than that in MGC-803/EV or MGC-803 cells.

suggest that Cdx2 suppresses cell proliferation by controlling the G1 and S checkpoints and inducing a specific block in cell cycle progression.

Cdx2 overexpression induces cellular apoptosis. To confirm that Cdx2 induces MGC-803 cell apoptosis, cells were stained with Annexin V-FITC and PI before analysis by flow cytometry. As indicated in Fig. 6, the percentage of MGC-803/Cdx2 cells that were Annexin V^+/PI^-, apoptotic fraction, was significantly higher than that of MGC-803/EV or MGC-803 cells ($P<0.05$). This finding implies that overexpression of Cdx2 could induce apoptosis in gastric cancer MGC-803 cells.

Cdx2 overexpression inhibits migration and invasion of gastric cancer cells. We measured the ability of three cell groups to migrate and invade using the wound healing assay and invasion assay system. A monolayer of cells was scratched, and the distance between the wound edges was determined at 0, 24, 48 and 72 h. MGC-803/Cdx2 cells showed lower migration ability at all time-points compared to both the controls ($P<0.05$). The relative migration of MGC-803/Cdx2 cells after 48 h was 59.6±5.3%, which was 23.7 and 28.2% less than that of MGC-803/EV and MGC-803 cells, respectively (Fig. 7).

After the cells were incubated for 24 h in the invasion assay system, the numbers of MGC-803/EV and MGC-803 cells that had moved through the membrane of the Matrigel chamber were about 2.7-fold higher than that of MGC-803/Cdx2 cells ($P<0.05$) (Fig. 8). The results indicate that Cdx2 reduces the migration and invasion ability of gastric cancer MGC-803 cells.

Table II. Cell cycle analysis by flow cytometry in gastric cancer MGC-803 cells.

Group	Cell cycle phase		
	G0/G1 (%)	S (%)	G2/M (%)
MGC-803 cells	33.9±3.2	52.3±5.5	13.8±6.1
MGC-803/EV cells	35.6±3.4	48.7±1.8	15.7±4.5
MGC-803/Cdx2 cells	47.1±6.4[a]	35.8±3.1[a]	17.1±3.5

[a]P<0.05, MGC-803/Cdx2 cells vs. MGC-803/EV cells and MGC-803 cells.

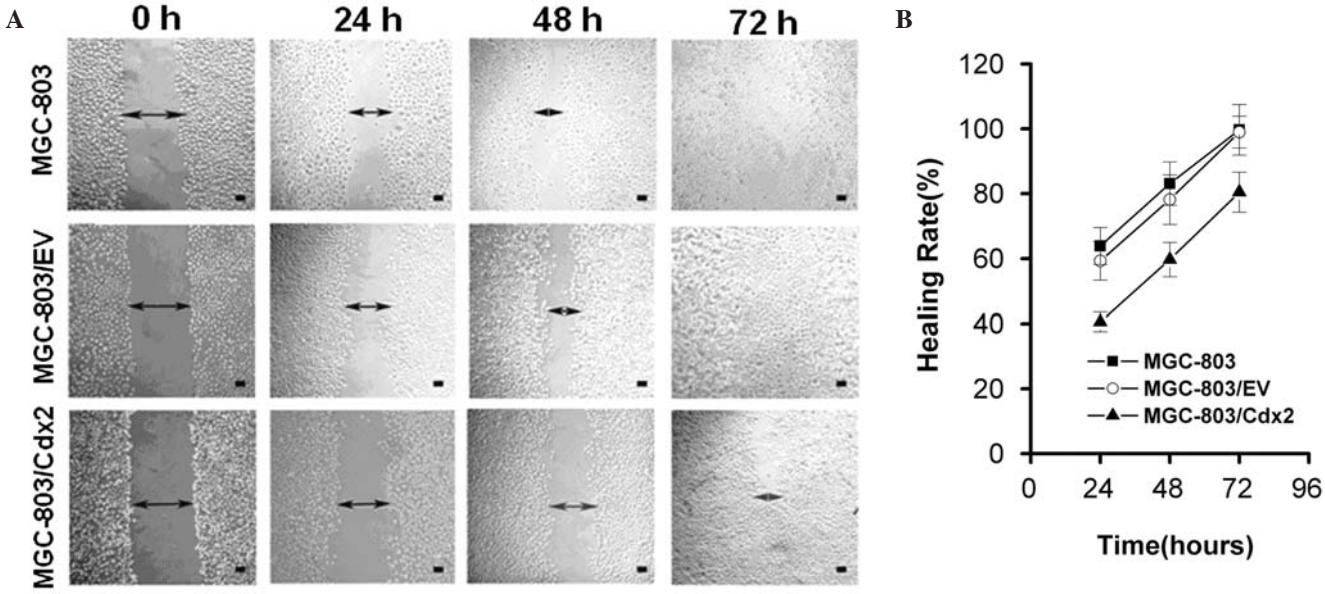

Figure 7. Results of wound healing assay of gastric cancer MGC-803 cells. (A) A representative result of cell migration. (B) The MGC-803/Cdx2 cells exhibited decreased migration compared with the corresponding controls (MGC-803/EV and MGC-803 cells) in wound healing assay.

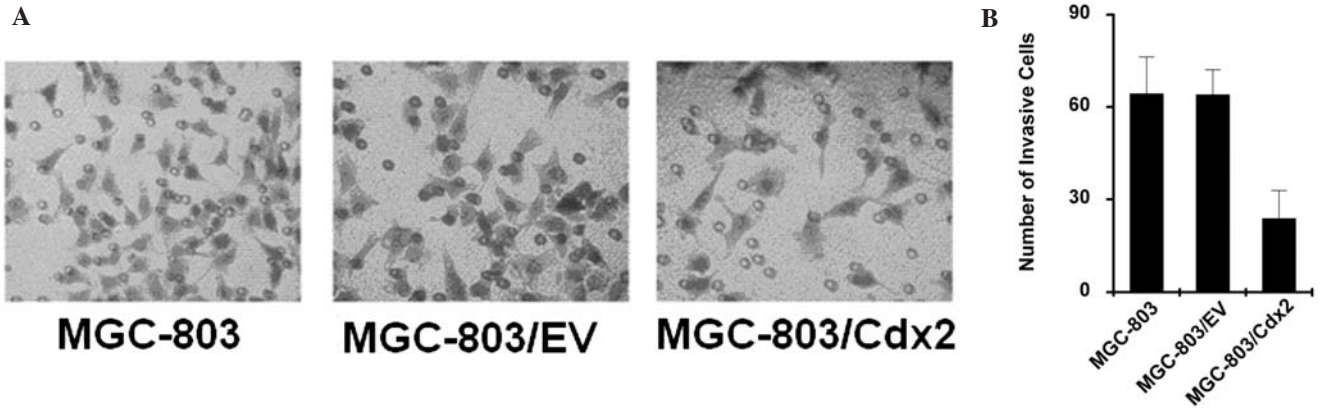

Figure 8. Results of invasion assay of gastric cancer MGC-803 cells. (A) A representative result of cell invasiveness obtained from three independent experiments. (B) The invading ability by counting the number of MGC-803/Cdx2 cells on the lower sides of the transwell under a microscope at 24 h was significantly reduced.

Discussion

Homeobox genes encode nuclear transcription factors involved in patterning and cell differentiation during development (13). They have also been identified as a new class of proto-oncogenes (4), and substantial evidence indicates that homeobox gene alterations participate in tumorigenesis (14). Considerable progress in understanding the function of homeobox genes arose from the finding that they regulate molecules involved in cellular interactions such as cell

adhesion molecules and extracellular matrix components (15). Homeobox genes themselves are regulated by other homeobox genes (16), retinoids (17) and/or growth factors (18).

The caudal-related homeobox gene Cdx2 encodes a homeodomain transcription factor required for development and maintenance of the intestinal epithelium (19,20). Cdx2 expression in adults is restricted to intestinal epithelial cells, where it maintains the differentiated phenotype of mature enterocytes by regulating expression of intestine-specific genes, including guanylyl cyclase C (GCC), lactase, sucrase-isomaltase and LI-cadherin (21-23). Also, Cdx2 regulates the expression of ubiquitous genes important in controlling cell dynamics, including adhesion, proliferation, cell cycle regulation and apoptosis (24-26). The homeotic function of Cdx2 is underscored by loss of the cognate enterocyte phenotype in intestinal neoplasms arising in the proximal colon of $Cdx2^{+/-}$ mice upon biallelic inactivation of Cdx2 (9,27). Conversely, ectopic expression of Cdx2 in squamous epithelial cells of the forestomach induces intestinal metaplasia in transgenic mice (28,29).

Beyond its homeotic role, Cdx2 may be an important tumor suppressor in carcinogenesis (2,30). Thus, expression of Cdx2 mRNA and protein is reduced or lost in some human tumors (31-34). However, in some analyses, immunohistochemistry revealed Cdx2 protein expression in most human colonic adenocarcinomas examined (3,35). In animal models, biallelic inactivation of Cdx2 results in the formation of hamartomatous tumors characterized by gastric heteroplasia in the proximal colon (36). Similarly, whereas adenomatous *polyposis coli* heterozygous ($APC^{+/-}$) mice develop adenomatous polyposis of the small intestine, $APC^{+/-}Cdx2^{+/-}$ mice develop polyposis of the colon (37). Moreover, the procarcinogen azoxymethane induced invasive adenocarcinoma of the distal colon in $Cdx2^{+/-}$ mice but not wild-type littermates (9). Further, overexpression of Cdx2 in human colon cancer cells induces a less malignant phenotype, inhibiting proliferation, invasion, and migration while promoting the expression of genes characteristic of mature enterocytes (20,26,32,38,39).

Thus, the prevalent paradigm suggests that Cdx2 is a tumor suppressor whose reduced expression and/or function contributes to initiation and progression of cancer (2,30,37). This hypothesis presumes that promotion of carcinogenesis reflects loss of Cdx2 expression and/or function in tumor cells relative to normal intestinal epithelium. In the present study, we constructed recombinant pCMV-Cdx2-HA plasmids and successfully transfected these plasmids into human gastric carcinoma MGC-803 cells. Our results clearly showed that Cdx2 overexpression in MGC-803 cells inhibited cell growth and blocked transition from G1 to S phase. We also showed that overexpression of Cdx2 induced apoptosis. Furthermore, Cdx2 overexpression in MGC-803 cells reduced cellular migration, invasion and colony formation, providing evidence that overexpression of Cdx2 suppressed tumor progression. This study provides experimental evidence that Cdx2 is a tumour suppressor gene involved in cancer progression in gastric cancer. This action is functionally and geographically distinct from its homeotic role during gut development.

Unlike tumor suppressor genes such as APC and p53, which act in the colon and also outside of the gut (40,41), Cdx2 is the intestine specific tumor suppressor. However, little is known about the mechanisms regulating Cdx2 gene transcription and the mechanisms causing its decreased expression in the progression to carcinomas. Both transcriptional and post-transcriptional mechanisms controlling Cdx2 levels are likely to play key roles in the determination of intestinal cell fate, differentiation and transformation. Cdx2 is likely to contain multiple phosphorylation sites that either positively or negatively modulate its activity in response to different signaling pathways. Two studies had already reported the occurrence of p38α and ERK-dependent phosphorylation of Cdx2 within the amino-terminal region, upstream of the DNA-binding homeodomain (42,43). Except that, Gross *et al* revealed a novel and important mechanism of regulation for the Cdx2 transcription factor (44). This occurs via phosphorylation of the Cdx2 protein at serine 281 by cyclin-dependent kinase 2 (Cdk2), a checkpoint cell cycle regulator, controlled by cyclins A and E at the G1/S and S/G2 transitions.

One characteristic feature of any malignant tumor is the ability of tumor cells to invade and migrate into surrounding and/or distal tissue. Adhesion molecules such as cadherins are considered important during the process of invasion or metastasis. Loss of E-cadherin expression in tumor cells is known to be associated with invasion and metastasis, as well as transition from adenoma to carcinoma. Recently, it was found that serum-soluble E-cadherin is a useful prognostic marker for gastric cancer. The relationship between Cdx2 and adhesion molecules was previously studied in human colon cancer cell lines (45-47). The Cdx2-overexpressing cell line displays high resistance to trypsin treatment at confluency, perhaps as a result of increased expression of E-cadherin in these cells. In the present study, we found inhibition of migration and invasion potential of MGC-803 cells *in vitro* because of the transfection of pCMV-Cdx2-HA plasmids.

In summary, the Cdx2 homeobox gene has multiple roles in the gut. During development it has a homeotic function and is an essential component of the gene complex required for midgut morphogenesis. The present data suggest that it exerts a homeostatic function in adults, whose overexpression inhibits tumor progression in human gastric carcinoma MGC-803 cells. Hence the tumor suppressor Cdx2 may be a putative target for anti-gastric cancer therapy, with the aim of restoring its expression to slow down or prevent malignant progression.

Acknowledgments

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References

1. Cillo C, Cantile M, Faiella A and Boncinelli E: Homeobox genes in normal and malignant cells. J Cell Physiol 188: 161-169, 2001.

2. Guo RJ, Suh ER and Lynch JP: The role of Cdx proteins in intestinal development and cancer. Cancer Biol Ther 3: 593-601, 2004.
3. Kaimaktchiev V, Terracciano L, Tornillo L, *et al*: The homeobox intestinal differentiation factor CDX2 is selectively expressed in gastrointestinal adenocarcinomas. Mod Pathol 17: 1392-1399, 2004.
4. Maulbecker CC and Gruss P: The oncogenic potential of deregulated homeobox genes. Cell Growth Differ 4: 431-441, 1993.
5. Soubeyran P, Andre F, Lissitzky JC, *et al*: Cdx1 promotes differentiation in a rat intestinal epithelial cell line. Gastroenterology 117: 1326-1338, 1999.
6. Almeida R, Silva E, Santos-Silva F, Silberg DG, Wang J, De Bolos C and David L: Expression of intestine-specific transcription factors, CDX1 and CDX2, in intestinal metaplasia and gastric carcinomas. J Pathol 199: 36-40, 2003.
7. Kim GH, Lee DH, Kim HW, *et al*: Relationships between the expressions of CDX1 and CDX2 mRNA and clinicopathologic features in colorectal cancers. Korean J Intern Med 20: 317-324, 2005.
8. Kim S, Domon-Dell C, Wang Q, *et al*: PTEN and TNF-alpha regulation of the intestinal-specific Cdx-2 homeobox gene through a PI3K, PKB/Akt, and NFkappaB-dependent pathway. Gastroenterology 123: 1163-1178, 2002.
9. Bonhomme C, Duluc I, Martin E, *et al*: The Cdx2 homeobox gene has a tumour suppressor function in the distal colon in addition to a homeotic role during gut development. Gut 52: 1465-1471, 2003.
10. Witek ME, Nielsen K, Walters R, Hyslop T, Palazzo J, Schulz S and Waldman SA: The putative tumor suppressor Cdx2 is overexpressed by human colorectal adenocarcinomas. Clin Cancer Res 11: 8549-8556, 2005.
11. Yang L: Incidence and mortality of gastric cancer in China. World J Gastroenterol 12: 17-20, 2006.
12. Xiao Q, Li L, Xie Y, *et al*: Transcription factor E2F-1 is up-regulated in human gastric cancer tissues and its overexpression suppresses gastric tumor cell proliferation. Cell Oncol 4: 335-349, 2007.
13. McGinnis W and Krumlauf R: Homeobox genes and axial patterning. Cell 68: 283-302, 1992.
14. Cillo C: HOX genes in human cancers. Invasion Metastasis 14: 38-49, 1994-1995.
15. Edelman GM and Jones FS: Outside and downstream of the homeobox. J Biol Chem 268: 20683-20686, 1993.
16. Hayashi S and Scott MP: What determines the specificity of action of Drosophila homeodomain proteins? Cell 63: 883-894, 1990.
17. Gudas LJ: Retinoids and vertebrate development. J Biol Chem 269: 15399-15402, 1994.
18. Pavlova A, Boutin E, Cunha G and Sassoon D: Msx1 (Hox-7.1) in the adult mouse uterus: cellular interactions underlying regulation of expression. Development 120: 335-345, 1994.
19. Suh E, Chen L, Taylor J and Traber PG: A homeodomain protein related to caudal regulates intestine-specific gene transcription. Mol Cell Biol l4: 7340-7351, 1994.
20. Suh E and Traber PG: An intestine-specific homeobox gene regulates proliferation and differentiation. Mol Cell Biol 16: 619-625, 1996.
21. Park J, Schulz S and Waldman SA: Intestine-specific activity of the human guanylyl cyclase C promoter is regulated by Cdx2. Gastroenterology 119: 89-96, 2000.
22. Fang R, Santiago NA, Olds LC and Sibley E: The homeodomain protein Cdx2 regulates lactase gene promoter activity during enterocyte differentiation. Gastroenterology 118: 115-127, 2000.
23. Hinoi T, Lucas PC, Kuick R, Hanash S, Cho KR and Fearon ER: CDX2 regulates liver intestine-cadherin expression in normal and malignant colon epithelium and intestinal metaplasia. Gastroenterology 123: 1565-1577, 2002.
24. Uesaka T, Lu H, Katoh O and Watanabe H: Heparin binding EGF-like growth factor gene transcription regulated by Cdx2 in the intestinal epithelium. Am J Physiol Gastrointest Liver Physiol 283: G840-G847, 2002.
25. Bai YQ, Miyake S, Iwai T and Yuasa Y: CDX2, a homeobox transcription factor, upregulates transcription of the p21/WAF1/CIP1gene. Oncogene 22: 7942-7949, 2003.
26. Mallo GV, Soubeyran P, LissitzkyJC, *et al*: Expression of the Cdx1 and Cdx2 homeotic genes leads to reduced malignancy in colon cancer-derived cells. J Biol Chem 273: 14030-14036, 1998.
27. Chawengsaksophak K, James R, Hammond VE, Kontgen F and Beck F: Homeosis and intestinal tumours in Cdx2 mutant mice. Nature 386: 84-87, 1997.
28. Mutoh H, HakamataY, Sato K, *et al*: Conversion of gastric mucosa to intestinal metaplasia in Cdx2-expressing transgenic mice. Biochem Biophys Res Commun 294: 470-479, 2002.
29. Silberg DG, Sullivan J, Kang E, *et al*: Cdx2 ectopic expression induces gastric intestinal metaplasia in transgenic mice. Gastroenterology 122: 689-696, 2002.
30. Abate-Shen C: Deregulated homeobox gene expression in cancer: cause or consequence? Nat Rev Cancer 2: 777-785, 2002.
31. Hinoi T, Tani M, Lucas PC, *et al*: Loss of CDX2 expression and microsatellite instability are prominent features of large cell minimally differentiated carcinomas of the colon. Am J Pathol 159: 2239-2248, 2001.
32. Hinoi T, Loda M and Fearon ER: Silencing of CDX2 expression in colon cancer via a dominant repression pathway. J Biol Chem 278: 44608-44616, 2003.
33. Okon K, Zazula M, Rudzki Z, Papla B, Osuch C and Stachura J: CDX-2 expression is reduced in colorectal carcinomas with solid growth pattern and proximal location, but is largely independent of MSI status. Pol J Pathol 55: 9-14, 2004.
34. Mallo GV, Rechreche H, Frigerio JM, *et al*: Molecular cloning, sequencing and expression of the mRNA encoding human Cdx1 and Cdx2 homeobox. Downregulation of Cdx1 and Cdx2 mRNA expression during colorectal carcinogenesis. Int J Cancer 74: 35-44, 1997.
35. Werling RW, Yaziji H, Bacchi CE and Gown AM: CDX2, a highly sensitive and specific marker of adenocarcinomas of intestinal origin: an immunohistochemical survey of 476 primary and metastatic carcinomas. Am J Surg Pathol 27: 303-310, 2003.
36. Beck F, Chawengsaksophak K, Waring P, Playford RJ and Furness JB: Reprogramming of intestinal differentiation and intercalary regeneration in Cdx2 mutant mice. Proc Natl Acad Sci USA 96: 7318-7323, 1999.
37. Aoki K, Tamai Y, Horiike S, Oshima M and Taketo MM: Colonic polyposis caused by mTOR-mediated chromosomal instability in Apc^{+}/ Delta716 Cdx2$^{+/-}$ compound mutant mice. Nat Genet 35: 323-330, 2003.
38. Brabletz T, Spaderna S, Kolb J, *et al*: Down-regulation of the homeodomain factor Cdx2 in colorectal cancer by collagen type I: an active role for the tumor environment in malignant tumor progression. Cancer Res 64: 6973-6977, 2004.
39. Lorentz O, Duluc I, Arcangelis AD, Simon-Assmann P, Kedinger M and Freund JN: Key role of the Cdx2 homeobox gene in extracellular matrix-mediated intestinal cell differentiation. J Cell Biol 139: 1553-1565, 1997.
40. Chung DC: The genetic basis of colorectal cancer: insights into critical pathways of tumorigenesis. Gastroenterology 119: 854-865, 2000.
41. Kinzler KW and Vogelstein B: Lessons from hereditary colorectal cancer. Cell 87: 159-170, 1996.
42. Houde M, Laprise P, Jean D, Blais M, Asselin C and Rivard N: Intestinal epithelial cell differentiation involves activation of p38 mitogen-activated protein kinase that regulates the homeobox transcription factor CDX2. J Biol Chem 276: 21885-21894, 2001.
43. Rings EH, Boudreau F, Taylor JK, Moffett J, Suh ER and Traber PG: Phosphorylation of the serine 60 residue within the Cdx2 activation domain mediates its transactivation capacity. Gastroenterology 121: 1437-1450, 2001.
44. Gross I, Lhermitte B, Domon-Dell C, *et al*: Phosphorylation of the homeotic tumor suppressor Cdx2 mediates its ubiquitin-dependent proteasome degradation. Oncogene 24: 7955-7963, 2005.
45. Keller MS, Ezaki T, Guo RJ and Lynch JP: Cdx1 or Cdx2 expression activates E-cadherin-mediated cell-cell adhesion and compaction in human COLO 205 cells. Am J Physiol Gastrointest Liver Physiol 287: G104-G114, 2004.
46. Ezaki T, Guo RJ, Li H, Reynolds AB and Lynch JP: The homeodomain transcription factors Cdx1 and Cdx2 induce E-cadherin adhesion activity by reducing beta- and p120-catenin tyrosine phosphorylation. Am J Physiol Gastrointest Liver Physiol 293: G54-G65, 2007.
47. Funakoshi S, Ezaki T, Kong J, Guo RJ and Lynch JP: Repression of the desmocollin 2 gene expression in human colon cancer cells is relieved by the homeodomain transcription factors Cdx1 and Cdx2. Mol Cancer Res 6: 1478-1490, 2008.