

# Homeobox D10 Gene, a Candidate Tumor Suppressor, Is Downregulated through Promoter Hypermethylation and Associated with Gastric Carcinogenesis

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Homeobox D10 (*HoxD10*) gene plays a critical role in cell differentiation and morphogenesis during development. However, the function of *HoxD10* in tumor progression remains largely unknown. We demonstrate that the expression of *HoxD10* is commonly downregulated in gastric cancer tissues (n = 33) and cell lines (n = 8) relative to normal stomach tissues. Functionally, reexpression of *HoxD10* results in significant inhibition of cell survival, induction of cell apoptosis, and impairment of cell migration and invasion. Moreover, ectopic expression of *HoxD10* suppresses gastric tumor growth in a mouse xenograft model. To identify target candidates of *HoxD10*, we performed cDNA microarray and showed that *HoxD10* regulates multiple downstream genes including IGFBP3. Reintroduction of *HoxD10* transcriptionally upregulates IGFBP3, activates caspase 3 and caspase 8, and subsequently induces cell apoptosis. Methylation specific PCR revealed that *HoxD10* promoter DNA was hypermethylated in gastric cancer cell lines. Additionally, 5-aza demethylation treatment could transiently reactivate the expression of *HoxD10* in gastric cancer cells. *HoxD10* promoter methylation frequently was detected in gastric cancer tissues obtained from endoscopic biopsies (85.7%, 24/28) and surgically resected samples (82.6%, 57/69). Intestinal metaplasia tissues showed a 60% methylation rate (18/30), but no detectable methylation in normal stomach tissues (0%, 0/10). Taken together, our results suggest that *HoxD10* functions as a candidate tumor suppressor in gastric cancer, which is inactivated through promoter hypermethylation.

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## INTRODUCTION

Gastric cancer is the second leading cause of cancer-related death worldwide (1,2). Most intestinal-type gastric cancers are thought to develop from precancerous

lesions (atrophic gastritis and intestinal metaplasia). Both genetic and epigenetic alterations contribute to this progression (2–4). The transcriptional silencing of tumor suppressor genes (TSGs) by pro-

moter methylation is a major epigenetic event in the origin of many cancers, including gastric cancer (5–7). Thus, identification of novel TSGs inactivated by promoter methylation will be of great importance in understanding the progression of gastric cancer, and could be utilized as biomarkers for the early detection of cancer (7–9). We and others have found that zinc finger of the cerebellum (ZIC1), fructose-1, 6-bisphosphatase-1 (FBP1), chromodomain helicase DNA binding protein 5 (CHD5), *protocadherin 10*, and *Klotho* genes were silenced epigenetically in gastric cancer (10–14). In the current study, we demonstrate *HoxD10* as another important TSGs which undergoes epigenetic modification.

The homeobox (Hox) superfamily genes, including *HoxD10*, encode tran-

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scription factors that control cell differentiation and morphogenesis during development (15,16). In addition to their roles in development, numerous Hox genes (*HoxB13*, *HoxA5* and *HoxC6*) have been found to be expressed aberrantly in a variety of solid tumors, including breast, colon and prostate cancers (16–20). Emerging evidence suggests that the expression of Hox genes is under epigenetic control (19–22). For example, *HoxA5* is suppressed in breast cancer through promoter methylation, and its suppression is correlated with the loss of p53 expression (20). Studies also demonstrate that CpG islands (CpGIs) in the promoters of Hox genes are commonly methylated in lung cancer (21,22). The dysregulation of Hox genes may affect various pathways that play critical roles in tumorigenesis and cancer metastasis (19). Evidence shows that ectopic expression of *HoxB13* in prostate and colon cancer cells can suppress tumor growth through downregulating of  $\beta$ -catenin-TCF pathway (17,23). It has been shown that *HoxD10* expression is reduced in both breast and endometrial tumors (24). Overexpression of *HoxD10* impairs breast tumor cells motility and invasiveness significantly, which indicates that *HoxD10* may serve as a tumor suppressor (25). However, the functional role of *HoxD10* in tumorigenesis, and the biological significance of its epigenetic control in gastric cancer have not been defined.

As transcription factors, Hox proteins can regulate the expression of multiple downstream genes. Genome-wide analysis has revealed that genes including *BMP7*, *FGFR2* and *PDGFRA* are regulated transcriptionally by *HoxC6* gene in prostate cancer cell lines (26). It was accepted that Hox genes could regulate the promoter of genes by binding consensus elements TTAT, TAAT and TTAC. Combined with microarray profiling, DNA binding site analysis and chromatin immunoprecipitation (ChIP) analysis identified that five genes are directly regulated by *HoxC8* in mouse embryo fibroblast cells (27). These candidate genes have been implicated in oncogenesis, cell pro-

liferation and cell apoptosis. Thus, identification of downstream targets of *HoxD10* genes in the cancer setting may shed light on the understanding of the mechanisms on tumorigenesis.

In this study, we provide evidence that promoter hypermethylation contributes to the downregulation of *HoxD10* in gastric cancer, and these methylation events frequently occur in precancerous lesions and gastric cancer tissues. Ectopic expression of *HoxD10* suppresses tumor growth *in vivo* and *in vitro*. Additionally, *HoxD10* regulates multiple downstream genes which participate in carcinogenesis by microarray analysis. Thus, our results suggest that *HoxD10* potentially functions as a tumor suppressor that is inactivated through promoter hypermethylation in gastric cancer.

## MATERIALS AND METHODS

### Cell Culture and Demethylation Treatment

Eight gastric cancer cell lines (AGS, BGC823, HGC27, MGC803, MKN28, MKN45, NCI-N87 and SGC7901) were obtained from Riken Gene Bank (Tsukuba, Japan) and American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured in RPMI 1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum and incubated at 5% CO<sub>2</sub>, 37°C and 95% humidity.

Tumor cells (AGS, MKN28, MKN45 and NCI-N87) were treated for 72 h with 5  $\mu$ mol/L 5-aza-2'-deoxycytidine (5-Aza) (Sigma, St Louis, MO, USA) for induction of demethylation. 5-Aza was replenished every 24 h. An equivalent concentration of the vehicle (DMSO) was used as the control. In addition, cells were cultured for another 48 h after withdrawal of the treatment.

### Clinical Samples

Ninety-three tissue biopsies, including gastric cancer (n = 33), intestinal metaplasia (IM) (n = 30), and normal stomach (n = 33), were obtained from endoscopic samples from Sir Run Run Shaw Hospi-

tal and Second Affiliated Hospital in Zhejiang University. In addition, another 69 surgically resected gastric cancer and adjacent nontumor tissues were obtained as described previously (11,12). Gastric cancers are classified according to Lauren Standard. The IM pathological status was evaluated by the updated Sydney system. All patients gave written informed consent, and the study protocol was approved by the Clinical Research Ethics Committee of Sir Run Run Shaw Hospital of Zhejiang University. All specimens were frozen immediately in liquid nitrogen and stored at –80° C until further processing.

### Total RNA and Genomic DNA Extraction, Bisulphite Treatment

Total RNA and genomic DNA were extracted with Trizol Reagent (Invitrogen) following manufacturer's instruction. The concentrations of RNA and DNA were quantified by Nanodrop 1000 (Nanodrop, Wilmington, DE, USA). Genomic DNA was bisulphite-treated with Zymo DNA Modification Kit (Zymo Research, Orange, CA, USA) according to the protocol provided by the manufacture.

### RT-PCR and Quantitative Real-Time PCR

Reverse transcription reaction was performed using 1  $\mu$ g of total RNA with Reverse Transcription System (Promega, Madison, WI, USA). The expression levels of *HoxD10* were determined by conventional polymerase chain reaction (RT-PCR) with GoTaq polymerase (Promega) and quantitative RT-PCR (qPCR) using SYBR Green Master Mix Kit (TaKaRa) in an ABI 7500 PCR system. Glyceraldehyde-3-phosphohate dehydrogenase (GAPDH) was used as an internal control, and the expression levels of *HoxD10* mRNA were determined using the 2<sup>- $\Delta\Delta$ Ct</sup> method. All primer sequences are listed in Supplementary Table 1.

### Methylation Specific PCR (MSP) Analysis

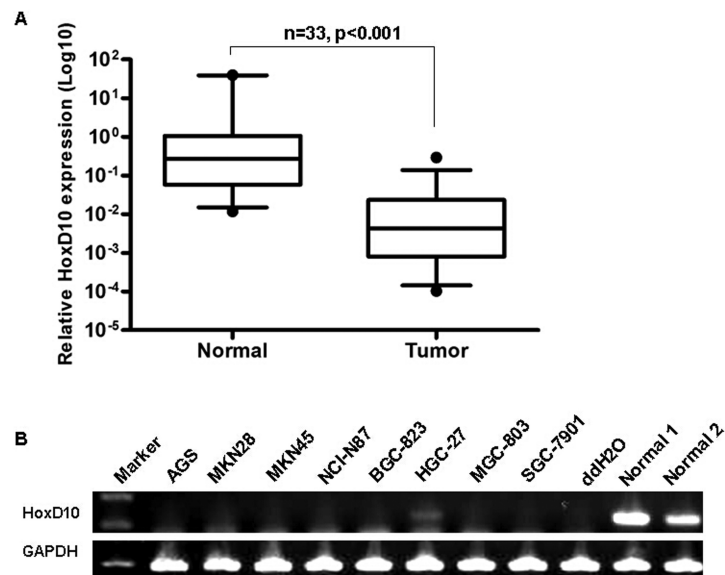
Promoter methylation of *HoxD10* was determined by methylation specific PCR

(MSP) using above bisulfite modified genomic DNA as the template. MSP was carried out for 40 cycles with an annealing temperature at 60° C as described previously (10). Methylation (M) primers were: F 5'-TTATT TATGG GGATT CGGGT TC, R 5'-AAAAA AACCG AATCC AAAAC AAACG, and Unmethylation (U) primers were: F 5'-ATTA TTTAT GGGGA TTTGG GTTT, R 5'-AAAAA AACCA AATCC AAAAC AAACA.

### Plasmid Construction and Cell Transfection

The *HoxD10* expression plasmid was constructed by cloning of the full-length *HoxD10* open reading frame into the mammalian expression vector pcDNA3.1 with *Bam*HI and *Xho*I restriction enzyme sites. The sequences were confirmed by DNA sequencing. Cells were cultured in 12-well plate for 24 h and transfected with pcDNA3.1-*HoxD10* or empty vector pcDNA3.1 using Lipofectamine 2000 (Invitrogen). G418 resistant colonies were selected in transfected cells (AGS and MKN28) for 14 d in a 12-well plate. After confirming the transfection efficiency by RT-PCR and Western blot in the surviving colonies, cells were transferred into a 6-well plate, and continuously selected with G418 to generate stable cell lines.

Two DNA fragments (–2,251 to –2,073 for BS1, and –1,727 to –943 for BS2) of *IGFBP3* were amplified by PCR using genomic DNA of HEK-293T cells as the template. The primer pairs were designed as follows, BS1 (Forward: GGGGT ACCCA TTCGG CACTG AACA A G, Reverse: GAAGA TCTAG CCTGG ACTGA CCACT G) and BS2 (Forward: GGGGT ACCGG CACTC CATTG TTCTT, Reverse: GAAGA TCTGA ATAAT AAAGA CAATA AACTG G). PCR products were digested with *Kpn*I and *Bgl*III enzymes and cloned into upstream of pGL3-promoter vector (Promega) to generate the recombinant plasmid of promoter-luciferase reporter gene. All constructs were confirmed by DNA sequencing.



**Figure 1.** Downregulation of *HoxD10* in gastric cancer. (A) *HoxD10* mRNA expression was determined by quantitative real-time PCR in 33 paired endoscopic biopsy samples from gastric cancer and normal stomach tissues. The data were described as relative  $\log_{10}$  value, and analyzed by Wilcoxon matched pairs test. (B) The expression profile of *HoxD10* was analyzed in eight gastric cancer cell lines, as well as in two of normal stomach tissues by conventional RT-PCR. GAPDH was used as the internal control. Normal: Normal stomach tissue.

### Colony Formation Assay

AGS and MKN28 cells were cultured for 24 h in a 12-well plate ( $1.5 \times 10^5$ /well) and transfected with pcDNA3.1 or pcDNA3.1-*HoxD10*. Forty hours later, the transfectants were replated in 6-well and selected with G418 resistant colonies for 14–16 d. Surviving colonies were stained with gentian violet after methanol fixation and visible colonies were counted. The experiments were performed in triplicate.

### Quantitative Chromatin

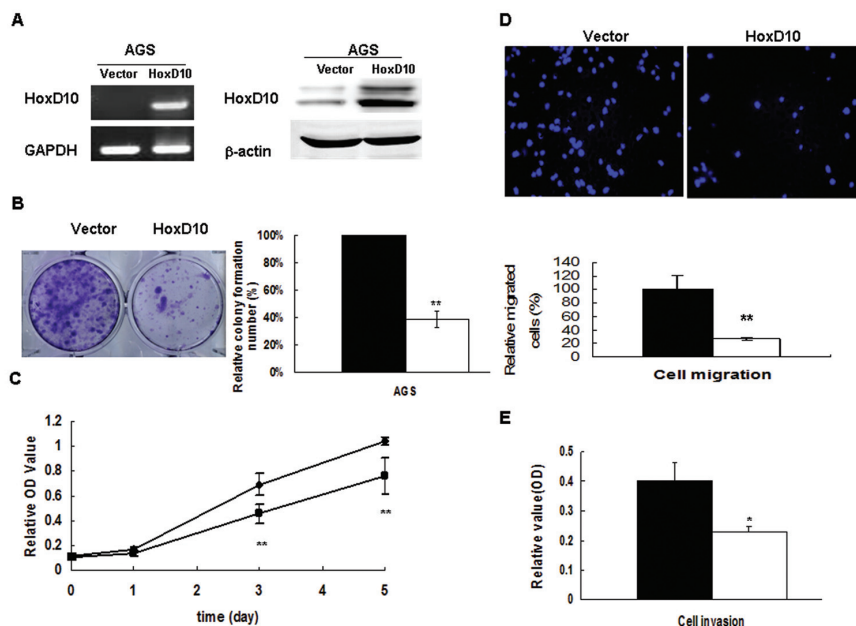
#### Immunoprecipitation (ChIP) Analysis

ChIP analysis was determined by the Simple ChIP Enzymatic Chromatin IP Kit (Cell Signaling Technology Inc., Danvers, MA). The chromatin was immunoprecipitated with normal rabbit IgG (negative control) and *HoxD10* monoclonal antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA). Quantitative real-time PCR was performed using SYBR Green Master Mix Kit (Takara Otsu, Shiga, Japan). According to transcription factor binding site prediction software (PROMO search program; Algorithmics and Genet-

ics Group [ALGGEN], Polytechnic University of Catalonia, Barcelona, Spain; <http://alggen.lsi.upc.es/>), five potential *HoxD10* binding sites (H1-H5) were localized in the upstream 2.3-kb sequence of *IGFBP3* gene. Four pairs of primers (A1-A4) were designed to detect different DNA fragments in the promoter regions of *IGFBP3* (A1 for H1 and H2, A2 for H3, A3 for H4 and A4 for H5, respectively) (Supplementary Table 2). The ChIP analysis results were expressed as fold change compare to rabbit IgG normalized to 1. The results were considered as positive *HoxD10*-binding sequences when the fold change greater than three-fold from triplicate experiments.

### Dual-Luciferase Reporter Assays

$5 \times 10^4$  Cells were cultured in 24-well plate for 24 h and transfected with pcDNA3.1-*HoxD10* or empty vector pcDNA3.1, pGL3-promoter vector or pGL3-BS (*IGFBP3*) and pRL-TK vector (Promega) containing reference control Renilla using Fugene HD (Roche, Penzberg, Germany). The activities of



**Figure 2.** *HoxD10* inhibits proliferation, and impairs cell migration and invasion of gastric cancer cells. (A) Overexpression of *HoxD10* in AGS gastric cancer cells was confirmed by RT-PCR (left lane) and Western blot (right lane). GAPDH and  $\beta$ -actin were used as internal controls. (B) The monolayer colony formation assays. AGS cells were transfected with pcDNA3.1-*HoxD10* or pcDNA3.1 empty vector and selected with G418 for 14 to 16 d. The numbers of survival colonies in each empty vector-transfected control were set to 100%, while *HoxD10*-transfected cells were presented as mean percentage  $\pm$  SD from three individual experiments in right diagram. (C) The number of viability cells was measured by MTS cell proliferation assays. The assay was performed at 6, 24, 72 or 120 h in AGS cells transfected with pcDNA3.1-*HoxD10* or pcDNA3.1 empty vector. Experiments were performed in triplicate. The asterisk indicates statistical significance (\* $P < 0.05$ , \*\* $P < 0.01$ ). (D) Cell migration assays were performed in modified Boyden transwell chambers assay.  $5 \times 10^4$  AGS cells/well were plated and incubated for 24 h. The migratory cells on the bottom of the membrane were fixed and nucleus stained with DAPI (upper figures). The mean number of visible cells was counted by fluorescence microscope in five random high power fields (lower bar diagram). (E) QC 24-Well Collagen-Based Cell Invasion Assay (Millipore, USA) was used to assess cell invasion. Invaded cells were stained with Cell Stain Solution, then detected on a standard microplate reader (560 nm). The asterisk indicates statistical significance (\* $P < 0.05$ , \*\* $P < 0.01$ ). B, E:  $\blacksquare$ , Vector;  $\square$ , *HoxD10*; C:  $\blacklozenge$ —, vector;  $\blacksquare$ —, *HoxD10*.

Firefly and Renilla luciferase were determined 30 h after the transfection by the dual-luciferase reporter assays (Promega) following the protocol of the manufacture. The relative fold of luciferase activity was expressed as firefly/renilla ratio. The experiments were performed in triplicate.

**Cell Proliferation Assay (MTS)**

Cell proliferation was detected with Non-Radioactive Cell Proliferation Assay with MTS reagents (Promega). Stable

transfected cells ( $1 \times 10^4$ /well) were plated into 96-well plates for 6, 24, 72 and 120 h respectively. After incubation with CellTiter 96 Aqueous One Solution reagent for 1 h, the absorbance was measured at 490 nm according to the manufacturer’s instruction.

**Xenograft Tumor Model**

Animal experimental protocols were approved by the Committee of Animal Ethics, Zhejiang University. Female athymic nude mice (nu/nu) (3 to 4 wks

old) were purchased from Shanghai Laboratory Animal Co Ltd (SLAC, Shanghai, China). MKN28 cells ( $7 \times 10^6$  cells) transfected with pcDNA3.1 or pcDNA3.1-*HoxD10* were injected subcutaneously into the flank of mice (n = 6 each group). After 6 d of implantation of tumor cells, tumor volume was assessed every 3 d. Tumor volume was calculated according to the following formula:  $V = L \times W^2 / 2$  where  $V$ , volume ( $\text{mm}^3$ );  $L$ , biggest diameter (mm);  $W$ , smallest diameter (mm). The mice were euthanized and the tumors were weighed at the end of the experiments.

**Cell Migration and Invasion Assay**

AGS cells transfected with pcDNA3.1 vector or pcDNA3.1-*HoxD10* were used for cell migration and invasion assays. Cell migration was assessed by modified Boyden transwell chambers assay (Corning, NY, USA). Briefly,  $5 \times 10^4$  cells/well were plated into 250  $\mu\text{L}$  of 5% FBS medium in the upper chamber, and 500  $\mu\text{L}$  of medium containing 15% FBS were added to the lower chamber. The cells were incubated for 24 h. The nonmigratory cells in the upper chamber were removed with a cotton swab. The cells on the bottom of the membrane were fixed and stained with DAPI. The number of visible cells was counted by fluorescence microscope in five random high power fields.

For evaluation of cell invasion, QCM 24-Well Collagen-Based Cell Invasion Assay (Millipore, Bedford, MA, USA) was used. Briefly, invaded cells on the bottom of the membrane were incubated with Cell Stain Solution, and 100  $\mu\text{L}$  of the dye mixture was transferred to a 96-well microtiter plate for colorimetric measurement at 560 nm.

**Cell Apoptosis and Cell Cycle Analysis**

Cell apoptosis assays were performed using the annexin V/PI kit (Invitrogen) by flow cytometry analysis (FCA). Briefly, stably transfected cells (AGS, MKN28) were suspended in annexin-binding buffer, Alexa Fluor 488 annexin V

and PI working solution were added in sequence. The stained cells were finally analyzed by FACSscan flow cytometry (BD, Franklin Lakes, NJ, USA). Meanwhile,  $2 \times 10^5$  seeded cells were exposed to ultraviolet light to induce apoptosis as a positive control.

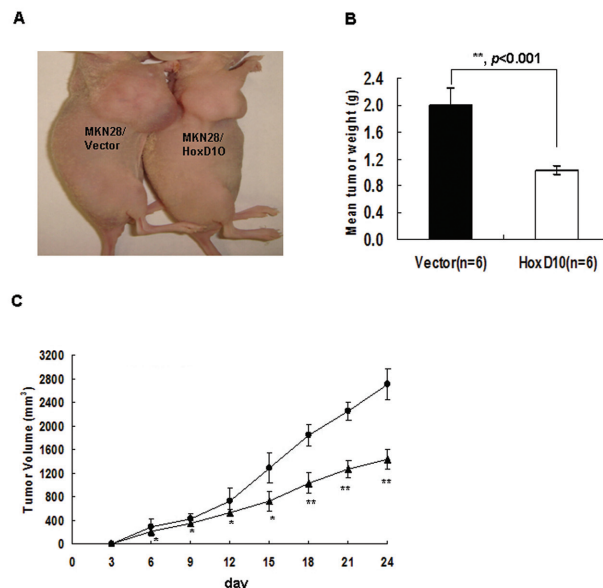
Cell cycle distribution was detected by the Cycletest Plus DNA Reagent kit (BD). Briefly, transfected cells were harvested and washed in PBS. Cellular DNA was stained with 125  $\mu\text{g}/\text{mL}$  propidium iodide for 20 min at 4° C in dark. The cells then were sorted by FACS Calibur, and cell-cycle distribution was determined using the ModFit LT software (Verity Software House Topsham, ME, USA).

### Western Blot Analysis

Total proteins were extracted from stably transfected cells using RAPI lysis buffer. Lysates were resolved on SDS-PAGE gel and transferred to PVDF membranes (Millipore). Primary antibodies were used as follows: HoxD10 (1:500; Santa Cruz Biotechnology Inc.), IGFBP3 (1:500; Santa Cruz Biotechnology Inc.), procaspase 3 (1:1000; Cell Signaling Technology Inc.), cleaved caspase 3 (1:1000; Cell Signaling Technology Inc.), procaspase 8 (1:1000; Cell Signaling Technology Inc.), cleaved caspase 8 (1:1000; Cell Signaling Technology Inc.) and  $\beta$ -actin (1:2500, Multisciences Biotech, Hangzhou, China). The blots were developed using a chemiluminescence with Las-4000 Imaging System (Fujifilm, Tokyo, Japan).

### cDNA Microarray

Total RNA was extracted from stably transfected MKN28 cells with pcDNA3.1-*HoxD10* or pcDNA3.1 vector, and reverse transcribed to cDNA. Labeled samples were hybridized to 41,000 probes and transcripts based on Affymetrix platform in duplicate. The microarray data was performed using Agilent Feature Extraction software. We selected  $\log_2$  ratio  $\geq 1$  or  $\leq -1$  as the threshold for upregulation or downregulation. The gene expression profiles were



**Figure 3.** *HoxD10* inhibits gastric tumor growth *in vivo*. MKN28 cells ( $7 \times 10^6$  cells) transfected with pcDNA3.1 vector or pcDNA3.1-*HoxD10* were injected subcutaneously into the flanks of athymic nude mice (3–4 wk old) mice ( $n = 6$  each group). (A) Representative pictures of tumor growth in nude mice inoculated subcutaneously with empty vector or *HoxD10* transfected cells at the end of the observation. (B) The tumor from each group was weighed immediately after removal. The average tumor weight is indicated as mean  $\pm$  SD. (C) After 6 d of implantation of tumor cells, tumor volume (mean  $\pm$  SD) was assessed every 3 d. The asterisk indicates statistical significance (\* $P < 0.05$ , \*\* $P < 0.01$ ). —●—, MKN28/vector; —▲—, MKN28/*HoxD10*.

classified into different clusters according to their functions (cell proliferation, apoptosis, migration and angiogenesis, for example). Nine representatives of target genes were verified with qPCR. All primers for qPCR are listed in Supplementary Table 1.

### Statistical Analysis

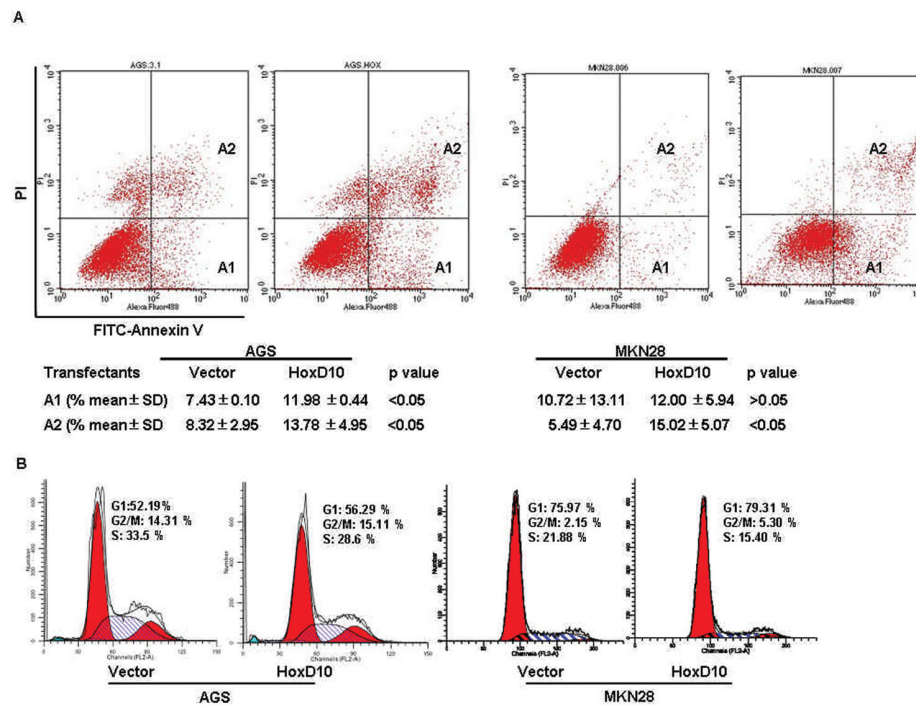
The differential *HoxD10* mRNA expression between gastric cancer and normal tissues was analyzed by the Wilcoxon matched pairs test. The two-tailed chi-square or Fisher exact tests were used for comparison of categorical variables. The probability of overall survival was calculated with the Kaplan-Meier method and differences between curves were evaluated with the Log-rank test. A cutoff of  $P < 0.05$  applied for statistical significance.

All supplementary materials are available online at [www.molmed.org](http://www.molmed.org).

## RESULTS

### *HoxD10* Is Downregulated in Gastric Cancer

We first determined *HoxD10* mRNA expression in 33 paired endoscopic biopsy samples from gastric cancer and normal stomach tissues (Supplementary Table 3). qPCR results showed that *HoxD10* expression was downregulated significantly in tumor tissues relative to normal gastric tissues ( $P < 0.001$ ) (Figure 1A). To further investigate the expression pattern of *HoxD10* in gastric cancer, we examined *HoxD10* expression in a panel of gastric cancer cell lines ( $n = 8$ ) by PCR and Western blot analysis. Results demonstrated that *HoxD10* expression was absent or reduced in all gastric cancer cell lines when compared to normal gastric tissues (Figure 1B and Supplementary Figure 1). Thus, these data suggest that *HoxD10* might function as a tumor suppressor in gastric cancer.



**Figure 4.** *HoxD10* induces cell apoptosis and cell-cycle inhibition. (A) The rate of cell apoptosis after stably transfected with *HoxD10* was determined by flow cytometry in AGS and MKN28 cell lines. Region A1 indicates early apoptotic cells, A2 shows late apoptotic cells. Experiments were performed in duplicate. (B) Cell-cycle distribution was analyzed by FACS flow cytometry in AGS and MKN28 cells stably transfected with pcDNA3.1-*HoxD10* or pcDNA3.1 vector. The mean percentages of cell-cycle phases from representative experiments are shown in each panel.

### *HoxD10* Suppresses Proliferation, Migration and Invasion of Gastric Cancer Cells

To understand the potential function of *HoxD10* in gastric cancer, AGS gastric cancer cells were transfected with pcDNA3.1-*HoxD10* or pcDNA3.1 vector. The transfection efficiency was confirmed by RT-PCR and Western blot (Figure 2A). We observed that the number of surviving colonies formed on the plates was significantly reduced in *HoxD10*-transfected cells when compared to the control vector transfectants ( $P < 0.01$ ) (Figure 2B). Similar results were reproduced in another gastric cell line MKN28 (Supplementary Figure 2). The cell proliferation inhibition of *HoxD10* in AGS cells was confirmed by MTS assay, which showed significant reduction in growth in *HoxD10*-transfected cells ( $P < 0.05$ ) (Figure 2C). In addition, ectopic expres-

sion of *HoxD10* suppressed AGS cell migration ( $P < 0.01$ , Figure 2D) and cell invasion ( $P < 0.01$ , Figure 2E) significantly in the 24-transwell cell migration and collagen-based cell invasion system, respectively. Collectively, these results indicate that ectopic expression of *HoxD10* inhibits gastric tumor cells survival, migration and invasion *in vitro*.

### *HoxD10* Inhibits Gastric Tumor Growth In Vivo

To further investigate the effects of *HoxD10* on gastric tumor growth, pcDNA3.1-*HoxD10* and pcDNA3.1 vector stably transfected MKN28 cells were injected subcutaneously into the flank of immunodeficient mice. The mean volume and wet weight of tumors in *HoxD10* transfectants ( $n = 6$ ) was significantly lower than that of the empty vector transfectants ( $n = 6$ ) at the end of the

experiment ( $P < 0.001$ ) (Figure 3A, 3B). In addition, tumor growth measured over a 3-wk period showed that tumor volume was decreased significantly in mice with ectopic expression of *HoxD10* relative to the empty vector group (Figure 3C). Thus, these results suggest that *HoxD10* inhibits tumor growth in gastric cancer *in vivo*.

### Ectopic Expression of *HoxD10* Induces Apoptosis and Cell-Cycle Alteration in Gastric Cancer Cell Lines

To explore the mechanisms underlying the inhibition of cell proliferation by ectopic expression of *HoxD10*, we assessed cell apoptosis and cell-cycle by flow cytometry. Reexpression of *HoxD10* significantly induced apoptosis of gastric cancer cells when compared to empty vector transfected cells in AGS and MKN28 cell lines (Figure 4A). Additionally, we observed that *HoxD10*-transfected cells (AGS and MKN28) showed lower S phase populations in comparison to the empty vector transfectants (Figure 4B).

### *HoxD10* Modulates Multiple Downstream Genes including IGF1BP3 and Apoptotic-Related Caspases in Gastric Cancer Cells

To identify potential downstream targets of *HoxD10* in gastric cancer cells, we performed cDNA microarray in MKN28 cells. Results demonstrated alterations of multiple gene expression by ectopic overexpression of *HoxD10*. According to gene functional analysis (<http://www.genecards.org>), these genes have been reported to participate in cell proliferation, invasion, apoptosis, cell cycle and angiogenesis (representative genes shown in Figure 5A and Table 1). qPCR analysis was further performed to validate the expression pattern in nine selected genes. Our results showed that HCLS1, ANP32A, PDGFRL, IGF1BP3 and CXCL9 were upregulated ( $> 2$ -fold change), while RAC2, NTS, KRT5 and TUSC3 were downregulated ( $< -2$ -fold change) by overexpression of *HoxD10* in MKN28 cells (Figure 5B, Supplementary Table 4).

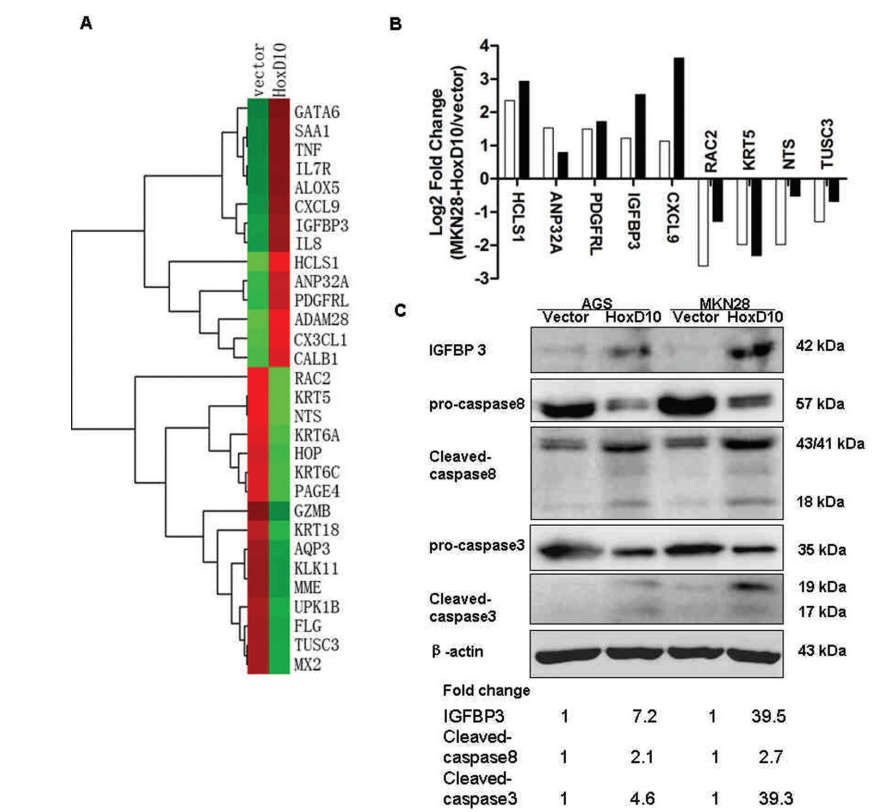
These results suggest that *HoxD10* potentially regulates downstream genes involved in tumorigenesis.

We further determined whether *HoxD10* modulates IGFBP3 and related apoptotic caspases in gastric cancer cells (AGS and MKN28). Consistent with microarray and qPCR analysis data, the expression of IGFBP3 protein was upregulated significantly by ectopic expression of *HoxD10* (Figure 5C, Supplementary Figure 3). It has been reported that IGFBP3 suppresses the growth of tumor cells (prostate and breast cancers) by activation caspases during cell apoptosis (28). Indeed, our results showed that reexpression of *HoxD10* leads to activating cleaved caspase 8 and caspase 3 (see Figure 5C). These results indicate that the induction of cell apoptosis by *HoxD10* may be mediated through IGFBP3 and subsequent activation of apoptotic caspases.

### *HoxD10* Transcriptionally Regulates the Expression of IGFBP3 in Gastric Cancer Cells

To determine whether *HoxD10* directly binds to the promoter of IGFBP3 gene, ChIP assay was adopted. We used the PROMO program to search for *HoxD10* transcription factor binding site in the putative IGFBP3 promoter. Five potential DNA sequences (H1-H5) were revealed as potential binding sites in the upstream 2.3-kb of IGFBP3 (Figure 6A). The ChIP assays were considered positive if the predicted *HoxD10*-binding sequence was enriched three-fold greater than the IgG antibody control. Quantitative PCR using the *HoxD10* antibody-precipitated chromatin showed that four predicted binding sites (A1, A2, A3 and A4) were enriched 2.5, 4.4, 12.7 and 7.1 times, respectively (Figure 6B). Results demonstrated that as a transcription factor, *HoxD10* could bind to multiple binding sites (A2, A3 and A4) of upstream IGFBP3.

To delve further into the *HoxD10* regulation of the transcription of IGFBP3, we have cloned two different DNA fragments (BS1 and BS2) in the promoter regions into a pGL3-promoter vector to



**Figure 5.** Gene expression profile changes by ectopic expression of *HoxD10*. (A) The gene expression profiles in *HoxD10* or control vector stable transfectants was visualized in MKN28 cell lines. Representative genes with over a two-fold change are indicated on the right side of this heatmap image. (B) The expression of nine genes was measured by qPCR and calculated using the value of  $2^{-\Delta\Delta CT}$ . Relative fold changes of *HoxD10* transfectants were compared with empty vector values normalized to 1. □, Result of cDNA microarray; ■, qRT-PCR data in MKN28 cells. (C) Western blot analysis of IGFBP3 and caspases. The expression of IGFBP3, caspase 3 (pro and cleaved) and caspase 8 (pro and cleaved) were detected after being transfected with *HoxD10* or empty vector in gastric cancer cell lines (AGS and MKN28). Band densities were normalized to  $\beta$ -actin. Relative fold change was expressed when compared with that of empty vector.

generate luciferase reporter gene (see Figure 6A). The luciferase reporter assays showed that when co-transfected with pcDNA3.1-*HoxD10* expression plasmid, luciferase activity of pGL3-BS2 promoter activity was enhanced by 4.0- and 4.4-fold respectively, when compared to gastric cancer cells (BGC823 and SGC7901) transfected with pcDNA3.1 vector ( $P < 0.01$ ). However, pGL3-BS1 promoter activity showed no significant change after being transfected with pcDNA3.1-*HoxD10* (Figure 6C). It should be emphasized that DNA fragments in *HoxD10* (BS2) have predicted IGFBP3 binding

sites (A2, A3 and A4), and BS1 fragments with A1 binding sites. These results indicate that *HoxD10* could directly bind to promoter sites (A2, A3 and A4), thus enhancing the expression of IGFBP3. Taken together, our results suggested that *HoxD10* transcriptionally regulates the expression of IGFBP3 in gastric cancer cells.

### Promoter Hypermethylation Contributes to *HoxD10* Gene Silencing in Gastric Cancer Cells

To address whether *HoxD10* is silenced though promoter hypermethylation

**Table 1.** Representative gene expression profile in *HoxD10* transfectants compared with empty vector control (fold change) by cDNA microarray

Gene symbol	Gene description	GenBank accession	Fold change	Gene function
<i>ADAM28</i>	ADAM metallopeptidase domain 28	NM_014265	3.84	Ad,P <sup>a</sup>
<i>ALOX5</i>	arachidonate 5-lipoxygenase	NM_000698	2.10	Ad,An,Ap,CC,M,PST
<i>ANP32A</i>	acidic (leucine-rich) nuclear phosphoprotein 32 family, member A	NM_006305	2.88	Ap,P
<i>AQP3</i>	aquaporin 3 (Gill blood group)	NM_004925	0.43	I,M,P
<i>CALB1</i>	calbindin 1, 28kDa	NM_004929	3.25	Ap,CC,M,ST
<i>CX3CL1</i>	chemokine (C-X3-C motif) ligand 1	NM_002996	3.50	Ad,An,Ap,M,P
<i>CXCL9</i>	chemokine (C-X-C motif) ligand 9	NM_002416	2.19	Ad,An,Ap,CC,I,M,PST
<i>FLG</i>	filaggrin	NM_002016	0.41	Ad,Ap,CC,I,M,P
<i>GATA6</i>	GATA binding protein 6	NM_005257	2.01	Ad,Ap,P
<i>GZMB</i>	granzyme B (granzyme 2, cytotoxic T-lymphocyte-associated serine esterase 1)	NM_004131	0.48	Ad,Ap,I,P
<i>HCLS1</i>	hematopoietic cell-specific Lyn substrate 1	NM_005335	5.10	Ap,M,PST
<i>GFBP3</i>	insulin-like growth factor binding protein 3	NM_001013398	2.33	Ad,An,Ap,CC,I,M,PST
<i>IL7R</i>	interleukin 7 receptor	NM_002185	2.11	Ad,Ap,CC,I,PST
<i>IL8</i>	interleukin 8	NM_000584	2.29	Ad,An,Ap,CC,I,M,PST
<i>KLK11</i>	kallikrein-related peptidase 11	NM_144947	0.43	Ap,M,P
<i>KRT18</i>	keratin 18	NM_000224	0.36	Ad,An,Ap,CC,I,M,PST
<i>KRT5</i>	keratin 5	NM_000424	0.25	Ad,Ap,I,P
<i>KRT6A</i>	keratin 6A	NM_005554	0.29	I,P
<i>MME</i>	membrane metallo-endopeptidase	NM_007289	0.43	Ad,An,Ap,CC,I,M,PST
<i>NTS</i>	neurotensin	NM_006183	0.25	Ad,An,Ap,CC,I,M,PST
<i>PDGFR</i>	platelet-derived growth factor receptor-like	NM_006207	2.82	PI
<i>RAC2</i>	Ras-related C3 botulinum toxin substrate 2 (rho family, small GTP binding protein Rac2)	NM_002872	0.16	Ad,Ap,CC,I,M,PST
<i>SAA1</i>	serum amyloid A1	NM_000331	2.07	Ad,An,Ap,I,M
<i>TNF</i>	tumor necrosis factor (TNF superfamily, member 2)	NM_000594	2.07	Ad,An,Ap,CC,I,M,PST
<i>TUSC3</i>	tumor suppressor candidate 3	NM_178234	0.41	Ap,CC,I
<i>UPK1B</i>	uroplakin 1B	NM_006952	0.40	ST

<sup>a</sup>Ad, adhesion; P, proliferation; An, angiogenesis; Ap, apoptosis; CC, cell cycle; M, migration; ST, signal transduction; I, invasion.

tion in gastric cancer, we examined the expression of *HoxD10* before and after treatment with 5-aza-2'-deoxycytidine (5-Aza) for 72 h. As shown in Figure 7A, the expression of *HoxD10* mRNA and protein was reactivated dramatically after 5-Aza treatment in gastric cancer cell lines (AGS, MKN28 and MKN45). While return of silencing expression of *HoxD10* was observed in MKN28 and MKN45 cells after withdrawing 5-Aza for another 48 h (Figure 7B). These suggested that *HoxD10* may be reactivated transiently after 5-aza treatment. Next, the methylation status of *HoxD10* in gastric cancer cells was determined by methylation-specific PCR. All tested gastric cancer cell lines showed full (MKN28, MKN45 and NCI-N87) or partial (AGS) methylation of *HoxD10* pro-

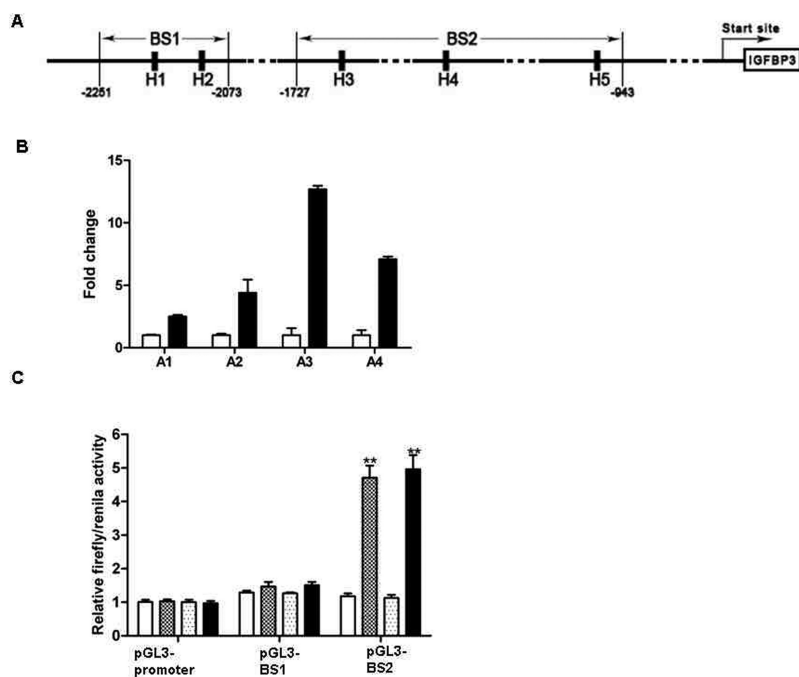
moter DNA (Figure 7C). These results suggest that DNA methylation may be associated with the transcriptional silencing of *HoxD10* in gastric cancer cells.

#### Promoter Hypermethylation of *HoxD10* Is Involved in Gastric Carcinogenesis

To further illustrate the relationship between *HoxD10* expression and promoter methylation, we investigated *HoxD10* methylation status in different endoscopic biopsy specimens from primary gastric cancers, atrophic gastritis with intestinal metaplasia and normal stomach tissues (Supplementary Table 4). The MSP results showed that the DNA promoter of *HoxD10* in primary gastric cancer biopsy tissues exhibited frequent methylation (85.7%,

24/28). Interestingly, as a precancerous lesion, atrophic gastritis with intestinal metaplasia tissues showed a relatively high rate of *HoxD10* methylation (60%, 18/30), whereas no methylation was observed in normal stomach tissues (0/10) (Figure 7D). To rule out the possibility that endoscopic biopsy specimens may not be representative of the whole tumor due to intratumor heterogeneity, we further evaluated the *HoxD10* methylation status in surgically resected samples from primary gastric cancers, as well as samples from adjacent nontumor tissues. Results illustrated that *HoxD10* promoter methylation was detected frequently in primary tumor samples (82.6%, 57/69), while most adjacent nontumor tissues did not demonstrate this methylation (see Fig-





**Figure 6.** *HoxD10* transcriptionally regulates the expression of IGFBP3 in gastric cancer cells. (A). Schematic representation of IGFBP3 putative promoter with *HoxD10* binding sites (BS). Upstream 2.3-kb sequence from the transcription start site of IGFBP3 were input to PROMO software to predict the possible *HoxD10* binding sites (H1-H5). IGFBP3 DNA fragments containing of *HoxD10* BS (-2,251 to -2,073 for BS1, and -1,727 to -943 for BS2) were cloned into pGL3-promoter vector and generated luciferase reporter gene. (B). Chromatin immunoprecipitation assays. The chromatin was immunoprecipitated with normal rabbit IgG and *HoxD10* antibody in BGC823 cells. Quantitative real-time PCR was performed to detect DNA fragments in the promoter regions of IGFBP3 (A1 for H1 and H2, A2 for H3, A3 for H4, A4 for H5 respectively). Results were expressed as fold change compared to rabbit IgG normalized to 1. The experiments were performed in triplicate. (C). Luciferase reporter gene assay. Gastric cancer cell lines (BGC823 and SGC7901) were transfected with pcDNA3.1-*HoxD10* or empty vector pcDNA3.1, pGL3-promoter vector or pGL3-BS, and pRL-TK vector. The relative fold of firefly activity was expressed normalized to renilla activity in pGL3-promoter vector. The experiments were performed in triplicate. \*\* $P < 0.01$  indicates of *HoxD10* versus empty vector. B: □, Rabbit IgG; ■, *HoxD10* antibody; C: □, BGC-vector; ▨, BGC-*HoxD10*; ▩, SGC-vector; ■, SGC-*HoxD10*.

ure 7D). We further analyzed the effect of *HoxD10* promoter methylation on survival of surgical gastric cancer patients using Kaplan-Meier survival analysis. *HoxD10* promoter hypermethylation was significantly associated with the lower survival times of gastric cancer patients ( $P = 0.028$ , Log-rank test) (Figure 7E). There was no significant correlation between the methylation of *HoxD10* promoter and clinicopathological features such as gender and Lauren type. Interestingly, *HoxD10* promoter hypermethylation seems to be

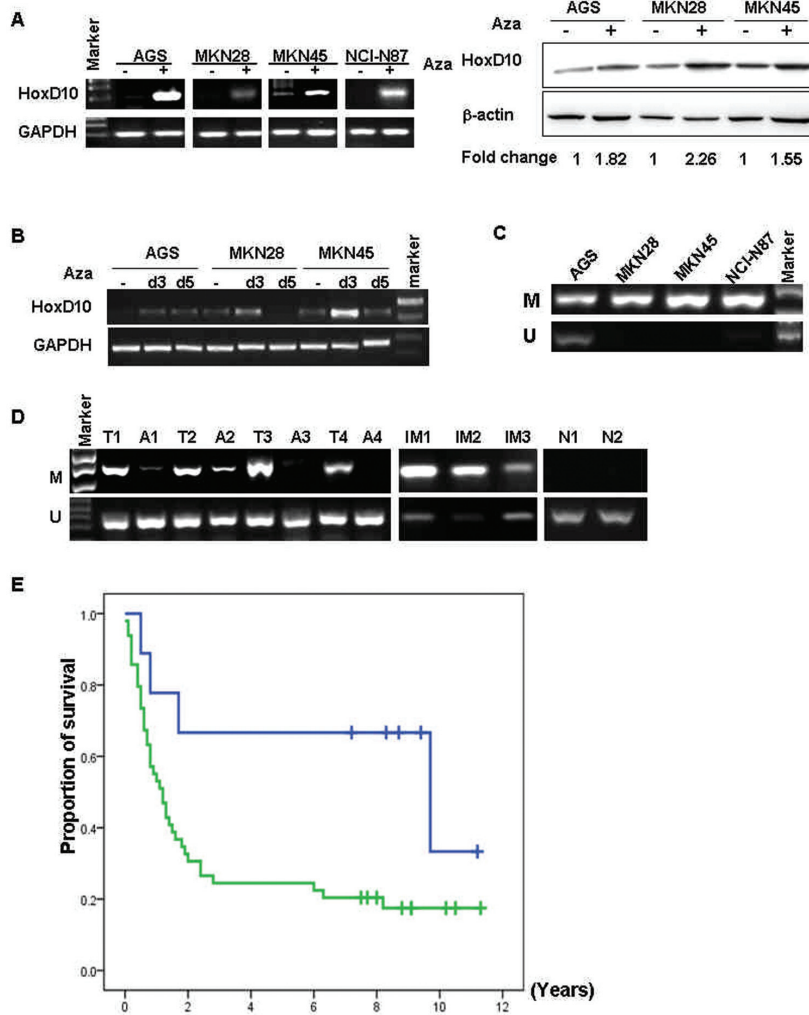
associated with the TNM stage and pathological differentiation in surgical gastric cancer patients (Supplementary Table 5). These results indicate that *HoxD10* promoter methylation contributes to the aberrant expression of *HoxD10*, and may be involved in gastric carcinogenesis.

## DISCUSSION

In this study, we demonstrated that downregulation of *HoxD10* is a common event during the gastric tumorigenesis. Ectopic expression of *HoxD10* inhibited

proliferation, impaired migration and invasion of gastric cancer cells. In particular, reexpression of *HoxD10* suppresses tumor growth in a mouse xenograft model. Taken together, our results suggest that *HoxD10* functions as a novel tumor suppressor in gastric cancer. *HoxD10* expression also was reported to be downregulated commonly other cancers including breast and endometrial tumors relative to normal tissues based on both *in situ* hybridization and RT-PCR analysis (24,25). Tumor growth suppression also was observed in prolactinoma with delivery of *HoxD10* using adenovirus/adenovirus-associated virus (Ad/AAV) (29). Collectively, the expression of *HoxD10* frequently is absent in cancer progression, and restoration of *HoxD10* might be an important strategy for gastric cancer therapy.

We revealed that demethylating treatment with 5-aza could restore the expression of *HoxD10* in gastric cancer cell lines (see Figure 7A). Furthermore, methylation of *HoxD10* promoter frequently was detected in gastric cancer cell lines, as well as in primary gastric cancer tissues (see Figure 7C, 7D). These findings strongly suggest that aberrant DNA methylation is one of the key mechanisms underlying *HoxD10* downregulation in gastric cancer. DNA hypermethylation has been viewed as a global process but only a few genes are functionally significant in tumorigenesis (5,7). DNA regions enriched with CpG dinucleotides, called CpG islands, once hypermethylated, can result in the silencing of tumor suppressor genes (TSGs) in cancer cells (5,6). Here, we identified *HoxD10* as another novel TSG inactivated though promoter hypermethylation in gastric cancer. It has been well reviewed that the paralogous *Hox* genes, including *HoxA5* and *HoxA7*, also are silenced epigenetically by promoter methylation in cancer progression (20,21,30), but we cannot exclude the appearance of other mechanisms in the silencing of *HoxD10* in gastric cancer. Another "epigenetic" modification by microRNAs (miRNAs) are encoded in *Hox*



**Figure 7.** DNA promoter of *HoxD10* is hypermethylated in gastric cancer. (A) *HoxD10* expression was examined in gastric cancer cells (AGS, MKN28, MKN45 and NCI-N87) after treatment with 5-Aza for 3 d (d3) by RT-PCR (left diagram) and Western blot (right diagram). (B) *HoxD10* expression was examined in gastric cancer cells (AGS, MKN28 and MKN45) after withdrawn of 5-Aza for another 2 d (d5). Aza: 5-aza-2'-deoxycytidine. (C) The methylation status of *HoxD10* promoter is detected by methylation-specific PCR (MSP) in the above gastric cancer cell lines. (D) The methylation status of *HoxD10* promoter in primary gastric cancer, intestinal metaplasia and normal stomach tissues was detected by MSP. Representative images are shown. T: Gastric tumor tissues; A: Adjacent nontumor tissues; IM: Intestinal metaplasia with atrophic gastritis tissues; N: Normal stomach tissue; M: Methylated; U: Unmethylated. (E) Survival curves were plotted based on Kaplan-Meier survival analysis. Methylation status of *HoxD10* promoter was used as the variate to separate two lines (n = 58, P = 0.028, log-rank test). —, Unmethylated; —, methylated.

cluster (19,31). Such miRNAs could regulate *Hox* gene expression by ways of mRNA cleavage or protein degradation (31,32). *HoxD10* is indeed an important target of miR-10b, and constitutive expression of *HoxD10* completely abrogated

miR-10b-induced cell motility and invasion in breast tumor (32). In addition, an intervening RNA, called *hox* transcript antisense intergenic RNA (*HOTAIR*), is emerging as a controlling factor of the expression of *HoxD* genes (33).

We attempted to search for novel downstream targets of *HoxD10* to identify pathways responsible for its tumor suppressing effect. Numerous genes are altered significantly by ectopic expression of *HoxD10* with cDNA microarray analysis. These candidate targets have been reported to participate in cellular growth, apoptosis, adhesion and angiogenesis (34–41). We further validated that *HCLS1*, *ANP32A*, *PDGFR*, *IGFBP3* and *CXCL9* were upregulated, while *RAC2*, *NTS*, *KRT5* and *TUSC3* downregulated by qPCR analysis. One gene of particular interest is *IGFBP-3*, which plays key roles on proliferation, migration and apoptosis in many cancers, including gastric cancer (28,34,35). Overexpression of *IGFBP-3* could induce cell apoptosis by activation of caspase 8 and caspase 3 (28). Our results revealed that ectopic expression of *HoxD10* could regulate *IGFBP3* transcriptionally by Chip and promoter luciferase analysis, and subsequent activation of caspase 3 and caspase 8. Furthermore, two other *HoxD10* downstream targets, *ANP32A* and *HCLS1*, also were involved in cell apoptosis. *ANP32A* is an important co-factor of caspase activation, which may enhance the activity of caspase 3 and determine sensitivity to apoptosis (36–38). *HCLS1* is reported to increase apoptosis by activation of caspase 3 activity (39). Thus, these results support our observation that *HoxD10* modulates several targets, at least including *IGFBP3*, *ANP32A* and *HCLS1*, that mediate caspase activation and induce cell apoptosis in gastric cancer. In addition to the regulation of apoptotic pathways, ectopic expression of *HoxD10* upregulates *CXCL9*, an inhibitor of tumor growth and metastasis (40). *RAC2*, Ras-related Rho GTPases, which was downregulated by *HoxD10* in our observation, enhances cell survival and migration (41). The identification of *HoxD10* target genes may facilitate our understanding of its functions as tumor suppressor.

To evaluate the potential utility of *HoxD10* as a biomarker in gastric oncogenesis *in vivo*, we examined the pro-

promoter methylation of *HoxD10* in primary gastric cancer tissues, as well as precancerous tissues. We observed that the *HoxD10* promoter DNA is hypermethylated in gastric cancer tissues obtained from endoscopic biopsies. Also, we found that *HoxD10* promoter hypermethylation was present in 82.6% (57/69) of surgically resected gastric cancer tissues, which removes the possibility of intratumor heterogeneity. Interestingly, precancerous lesions tissues showed a 60% (18/30) rate of *HoxD10* promoter hypermethylation relative to no methylation in normal stomach tissues (0/10). These results suggest that *HoxD10* methylation is a frequent event in gastric cancer and its early involvement in gastric carcinogenesis. Recent studies have shown that although the abnormal epigenetic silencing of genes can occur at any time during tumor progression, it occurs most frequently during the early stages of the neoplastic process, as well as during the precancerous stage (42,43). We and others have shown previously that many genes were silenced frequently through promoter hypermethylation in precancerous lesions such as intestinal metaplasia (12,14,44). These epigenetic alterations might initiate the expansion of premalignant cells which accumulate during the early stages of tumorigenesis (7,12,44,45). We also demonstrated that the *HoxD10* promoter hypermethylation was associated significantly with poor survival in gastric cancer patients. Our results indicate that the *HoxD10* promoter hypermethylation may be associated with gastric carcinogenesis and potentially utilized as a biomarker for the progression of gastric cancer.

## CONCLUSION

In summary, we identified *HoxD10* as a candidate tumor suppressor gene which is epigenetically silenced in gastric cancer.

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## DISCLOSURE

The authors declare that they have no competing interests as defined by *Molecular Medicine*, or other interests that might be perceived to influence the results and discussion reported in this paper.

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