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RESEARCH

Effects of Homeodomain Protein CDX2 Expression on the Proliferation and Migration of Lovo Colon Cancer Cells

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Abstract The homeobox gene, *CDX2*, plays a major role in development, especially in the gut, and also functions as a tumor suppressor in the adult colon. In the present study, we investigated the effects of CDX2 expression on the proliferation, migration, and apoptosis of the human colon cancer cell line, Lovo. Lovo cells exogenously expressing CDX2 exhibited no significant differences in the percentage of cells in G1- and S-phase or in apoptosis, as determined by flow cytometry. MTT assay also confirmed that CDX2 expression had no effect on proliferation in these cells. Interestingly, conditioned medium collected from CDX2-overexpressing Lovo cells showed a significant decrease in secretion of MMP-2 and the invasive potential of these cells was significantly inhibited. Collectively, these data suggest that CDX2 may play a critical role in the migration and metastasis of colon carcinoma and over-expression of CDX2 in colon cancer cells markedly inhibits invasion. Based on these results, exogenous expression of CDX2 might be a promising option in the treatment of colon carcinoma.

Keywords Proliferation · Invasion · CDX2 · Transfection · Colon cancer

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Abbreviations

CDX2	caudal-related homeobox protein 2
MTT	3-(4,5-dimethylthiazol-2yl)-2, 5-diphenyltetrazolium bromide
PI	propidium iodide
EMT	epithelial-mesenchymal transition

Introduction

Colorectal cancer (CRC) is one of the most common malignancies worldwide and is the second leading cause of cancer death in the United States [1]. However, despite many efforts, the molecular pathways involved and the order of genetic events in the genesis of CRC have not yet been clearly elucidated. Caudal-related homeobox protein 2 (CDX2) is expressed in intestinal epithelial cells distal to the duodenum and is critically involved in the development, proliferation, and differentiation of these cells [2]. Insight into the role of CDX2 in defining intestinal identity has been gained by examination of phenotypes resulting from CDX2 haploinsuffiency and gain-of-function mouse models. In adult tissue, a number of studies have identified the involvement of CDX2 in regulating the expression of genes encoding intestine-specific proteins, such as sucrase-isomaltase [3], lactase [4, 5], calbindin-D9K [6, 7], apolipoprotein B [8], claudin-2 [9], and MUC2 [10, 11]. Additionally, it has been demonstrated that CDX2 functions as a tumor suppressor in the adult colon. Bonhomme et al. showed that reduced expression of CDX2 accelerates tumor progression in a mouse model of sporadic colorectal cancer [12]. Furthermore, Aoki et al. confirmed these findings in a mouse model of familial adenomatous polyposis [13]. The role of CDX2 as a tumor suppressor is also supported by the observation

that its expression is decreased in human CRC in relation to tumor grade and in chemically-induced rat tumors [14–16]. Altered expression of homeobox genes, which leads to changes in cell migration and spreading, has been reported for several cancer types [17–20]. Histopathological analyses have demonstrated that CDX2 expression is low in invasive colorectal cancer cells, which localize at the tumor/stroma interface, but is restored in metastases at a level corresponding to the primary tumors [21]. These data suggest that decreased CDX2 expression plays a role in tumor migration.

In this study we designed a series of experiments in order to determine the effects of CDX2 expression on the proliferation and invasion of Lovo cells.

Materials and Methods

Cell Culture

The human colon cancer cell line, Lovo, was purchased from Type Culture Collection of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in RPMI 1640 medium (Gibco Biocult, Paisley, UK) containing 10% fetal bovine serum, 100 units/mL of penicillin, and 100 mg/mL of streptomycin at 37°C in a humidified 5% CO_2 atmosphere.

Plasmid Construction

CDX2 cDNA was cloned from a cDNA pool derived from the epithelium mucosae of normal human colon by reverse transcription polymerase chain reaction (RT-PCR), performed using the following primers: sense, 5′-ccaataagcttAGGCAG CATGGTGAGGTCTG-3′, and antisense, 5′-ccaatggatccCT GAGGAGTCTAGCAGAGTCCAC-3′, containing HindII and BamHI restriction sites, respectively (lower-case sequences). The 1,055 bp PCR product was subsequently extracted from the agarose gel and cloned into the eukaryotic expression vector, pEGFP-C1 (Clontech, Mountain View, CA) to generate pEGFP-C1-CDX2. CDX2 cloning was confirmed by restriction enzyme digestion and sequencing.

Transient Transfection of Lovo Cells

Transient transfection was carried out using the Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Briefly, $1.5–2\times10^5$ Lovo cells were seeded in each well of a 6-well tissue culture plate. The cells were incubated until 80% confluence. For each well, 4 μg purified plasmid mixed with 10 μL Lipofectamine 2000 in 2 ml serum-free RPMI 1640 medium was used. Six hours later the medium was replaced by complete medium. The cells were further incubated in 5% CO_2 at 37°C and harvested for analysis at different time points. Untreated cells and cells treated with control vector, pEGFP-C1, were used for comparison.

Western Blot Analysis

Approximately 1×10^7 Lovo cells were harvested by scraping 48 h after transfection, washed once with ice-cold PBS, resuspended in 100–200 μl lysis buffer (50 mM Tris, 150 mM NaCl, 5 mM EDTA, 5 mM EGTA, 1% SDS, pH=7.5), and then ultrasonicated on ice until the solution became clear. Total protein concentration was measured using the Bradford method according to the manufacturer's protocol (Sigma-Aldrich, St. Louis, MO). Samples were heated at 100°C for 5 min with and equal volume of 2X SDS loading buffer (125 mM Tris-HCl pH=6.8, 10% Glycerol, 2% SDS, 5% 2-Mercaptoethanol, 2.5 ml 0.0025% bromophenol blue) and briefly cooled on ice. Total protein (80 μg) from each sample was resolved by 8% or 10% SDS-PAGE to detect CDX2 (38 kD) and β-actin (43 kDa), respectively. Protein was then transferred to PVDF membrane in transfer buffer (25 mM Tris (pH=8.5), 200 mM glycerin, and 20% methanol) at 100 V for 2 h. Proteins were detected using mouse monoclonal antibody CDX2 (AM392-M, 1:500, BioGenex, San Ramon, CA) and mouse monoclonal antibody to β-actin (sc-47778, 1:1000, Santa Cruz, Santa Cruz, CA) as a loading control. Membranes were incubated with primary antibodies overnight at 4°C after blocking in 5% nonfat milk for 1 h at room temperature, and then incubated with horseradish peroxidase-conjugated goat anti-rabbit or goat anti-mouse IgG (BeiJing ZhongShan Goldbridge Biotechnology Co, Beijing, China). Proteins were visualized with chemiluminescence luminol reagents (sc-2048, Santa Cruz. Biotechnology, Santa Cruz, CA).

MTT Assay

The growth of Lovo cells was measured by Methyl thiazolyl tetrazolium (MTT) assay. Cells were plated onto 96-well plates at a density of 5×10^3 cells per well and left untreated or transfected with pEGFP-C1or pEGFP-C1-CDX2 as described above. The cells were incubated in 5% CO_2 at 37°C for 18 h, 42 h, and 66 h post-transfection. Subsequently, 20 ml of MTT reagent (Sigma; 5 mg/ml) was added to the medium and the reaction was allowed to proceed for 4 h at 37°C. Subsequently, Dimethyl Sulphoxide (DMSO; 150 μl) was added to each well to quench the reaction, the plate was shaken on a rotary platform for 10 min, and then the absorbance measured at a wavelength of 492 nm using a Dynatech MR 5000 multiscanner autoreader (Dynatech Industries. Inc., McLean, VA). The ratio of the absorbance of treated cells relative to that of the

control cells was calculated and expressed as a percentage of cell death.

Cell Cycle and Apoptosis Analysis

Approximately 5×10^5 Lovo cells per well were transfected with the appropriate plasmids, and 48 h later digested with 0.25% trypsin and harvested (Gibco BRL, Grand Island, NY). Cells were subsequently harvested and gently fixed (drop by drop) by the addition of 70% ethanol/PBS at 4°C overnight and cells being prepared for cell cycle analysis were then re-suspended in 200 μl PBS containing 40 μg/ml propidium iodide (PI) (Abcam, Cambridge, MA), 0.1 mg/mL RNase and 0.1% Triton X-100 in a dark room. After incubation at 37°C for 30 min, the cells were analyzed and cell cycle stage determined using a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, CA) equipped with an argon ion laser at a wavelength of 488 nm. Lovo cells being prepared for apoptosis analysis were also trypsinized and collected after pretreatment for 48 h. Cells were incubated in a binding buffer containing FITC-conjugated Annexin V and PI at room temperature for 5 min in the dark, according to the manufacturer's protocol (BD Biosciences). The percentage of apoptotic cells were then determined using a FACS Calibur flow cytometer. Experiments were conducted at least in triplicate.

Enzyme-linked Immunosorbent Assay (ELISA)

To determine the effect of CDX2 expression on the secretion of matrix metalloproteinase-2 (MMP-2) in tissue culture medium, the levels of MMP-2 in CM collected from Lovo cells was measured using commercially available ELISA kits from XiTang Biotechnology (F01860 XiTang

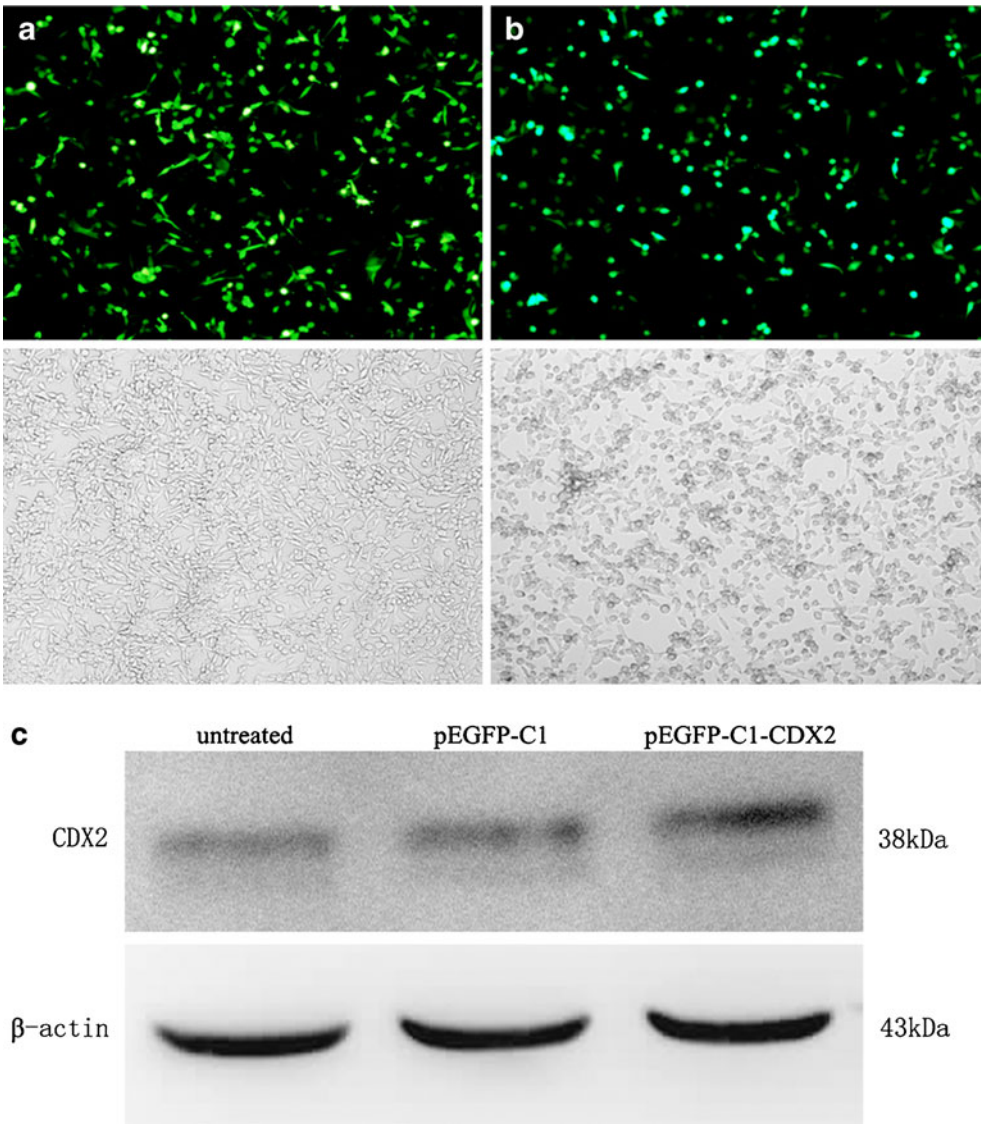

Fig. 1 Efficiency of transient CDX2 transfection in Lovo colon cancer cells. Lovo cells transfected with empty vector, pEGFP-C1 (**a**) or pEGFP-C1-CDX2 (**b**) were visualized with fluorescence microscopy (upper panels, 200×) and optical microscopy (lower panels, 200×). **c** Western blot analysis for CDX2 in untreated, pEGFP-C1-transfected and pEGFP-C1-CDX2-transfected cells

Biotechnology Co, Shanghai, China). Briefly, Lovo cells were left untreated or transiently transfected with pEGFP-C1 and pEGFP-C1-CDX2 as described above. The medium was replaced with serum free RPMI 1640 medium 6 h post-transfection, and the medium was collected at various time points (18 h, 42 h, and 66 h). The levels of MMP-2 protein secreted by the cells in the medium were determined by ELISA. In brief, 200 μl of sample or standard (recombinant human MMP-2) was added to the microplate wells with the recommended diluents and incubated at room temperature for 2 h. The wells were washed and 200 μl of MMP-2 conjugate (monoclonal antibody against MMP-2 conjugated to horseradish peroxidase) was added. Incubation was performed for 2 h, plates were washed, and then developed using the substrate solution (equal parts of H_2O_2 and tetramethylbenzidine). The reaction was subsequently stopped and the optical density read at 450 nm using a Dynatech MR 5000 multiscanner autoreader. The results were normalized to the number of cells per plate.

Establishment of Stable CDX2-overexpressing Lovo Cells

Lovo cells were plated into 6-well plates ($1.5–2\times10^5$ cells/well) and transiently transfected with pEGFP-C1 and pEGFP-C1-CDX2 as described above. After transient transfection for 48 h the cells were passaged at 1:10 (volume/volume) and cultured in medium supplemented with geneticin (G418) at 600 μg/mL for 8 wks. The survival clones were selected and maintained in medium containing 300 μg/mL G418.

Immunocytochemistry

Immunocytochemistry was performed using the streptavidin–peroxidase technique. Briefly, untreated, stably pEGFP-C1-transfected and pEGFP-C1-CDX2-transfected cells (stably expressing exogenous CDX2 cDNA) were plated on 8-mm coverslips with axenic medium and incubated overnight. Cells were then fixed with 4% paraformaldehyde in phosphate-buffered saline (PBS) and washed in 0.1% Triton X-100/PBS (pH=7.6) for 20 min at room temperature. Endogenous peroxidases were blocked by incubating with 0.3% H_2O_2 for 15 min at room temperature. The sections were incubated for 30 min at 37°C with normal nonimmune serum and then incubated overnight at 4°C with anti-E-cadherin monoclonal antibody (1:100, rabbit monoclonal; Boster Biological Technology, Wu Han, China). After washing three times with PBS, the sections were treated with biotin-conjugated secondary antibody and then streptavidin–peroxidase (Beijing Zhong Shan-Golden Bridge Biological Technology Co., Ltd.) was added. To visualize the colored reaction, diaminobenzidine (DAB) was applied and the protein was stained brown. The primary antibody was replaced by PBS in the negative controls.

In Vitro Invasion Assay

Polycarbonate membranes with 8.0 μm pore size were spread with 50 μL (10 μg) of Matrigel (BD Biosciences) on the upper surface of transwell cell culture chambers (Costar 3422, Cambridge, MA, USA) and incubated at 37°C. After 1 h, each insert was placed in 500 μL RPMI 1640 containing 0.1% BSA at 37°C for 30 min for hydration. The membranes were precoated with 50 μL (2 μg) fibronectin (Invitrogen) on the lower surface of the filters. The filters were dried at room temperature for 45 min. Untreated, pEGFP-C1-transfected, and pEGFP-C1-CDX2-transfected Lovo cells (1×10^5) were then suspended in 100 μL RPMI 1640 containing 0.1% FBS, added to the upper compartment, and incubated at 37°C for 24 h. The filters were fixed with 95% ethanol for 15 min and stained with crystal violet. After gently rinsing with water, the cells on the upper surface of the filters were removed by wiping with a cotton swab. The stained cells that invaded to the lower surface were photographed and manually counted under an optical microscope (×200 magnification). Each assay was performed in triplicate.

Statistical Analysis

All experiments were repeated at least three times. Results are expressed as mean ± standard deviation (SD). Significance was defined as $P<0.05$.

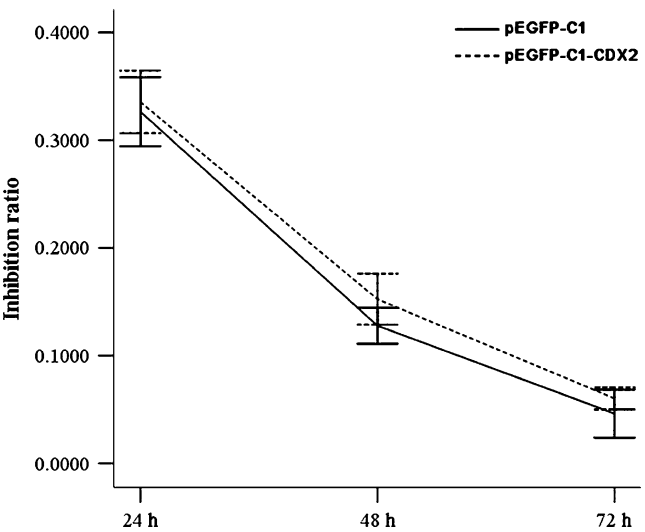

Fig. 2 Effects of pEGFP-C1-CDX2 on cell proliferation. Lovo cells were transfected with pEGFP-C1 or pEGFP-C1-CDX2 for 24 h, 48 h, and 72 h. The inhibition rate of the cells was then measured by MTT assay. Results are shown as the absorbance value at 492 nm

Results

Efficiency of Transiently Transfected pEGFP-C1 and pEGFP-C1-CDX2 in Lovo Colon Cancer Cells

Transient transfection of Lovo colon cancer cells with either pEGFP-C1 or pEGFP-C1-CDX2 plasmid was performed using Lipofectamine-2000. The transfection efficiency was determined by visualizing EGFP expression using fluorescence microscopy; the transfection efficiency was approximately 40% for both control and CDX2-expressing plasmids (Fig. 1a and b). CDX2 protein levels were significantly higher in pEGFP-C1-CDX2-transfected cells than in pEGFP-C1-transfected and untreated cells (Fig. 1c). Similar results were obtained when Lovo cells were stably transfected with control or CDX2-expressing plasmids (results not shown).

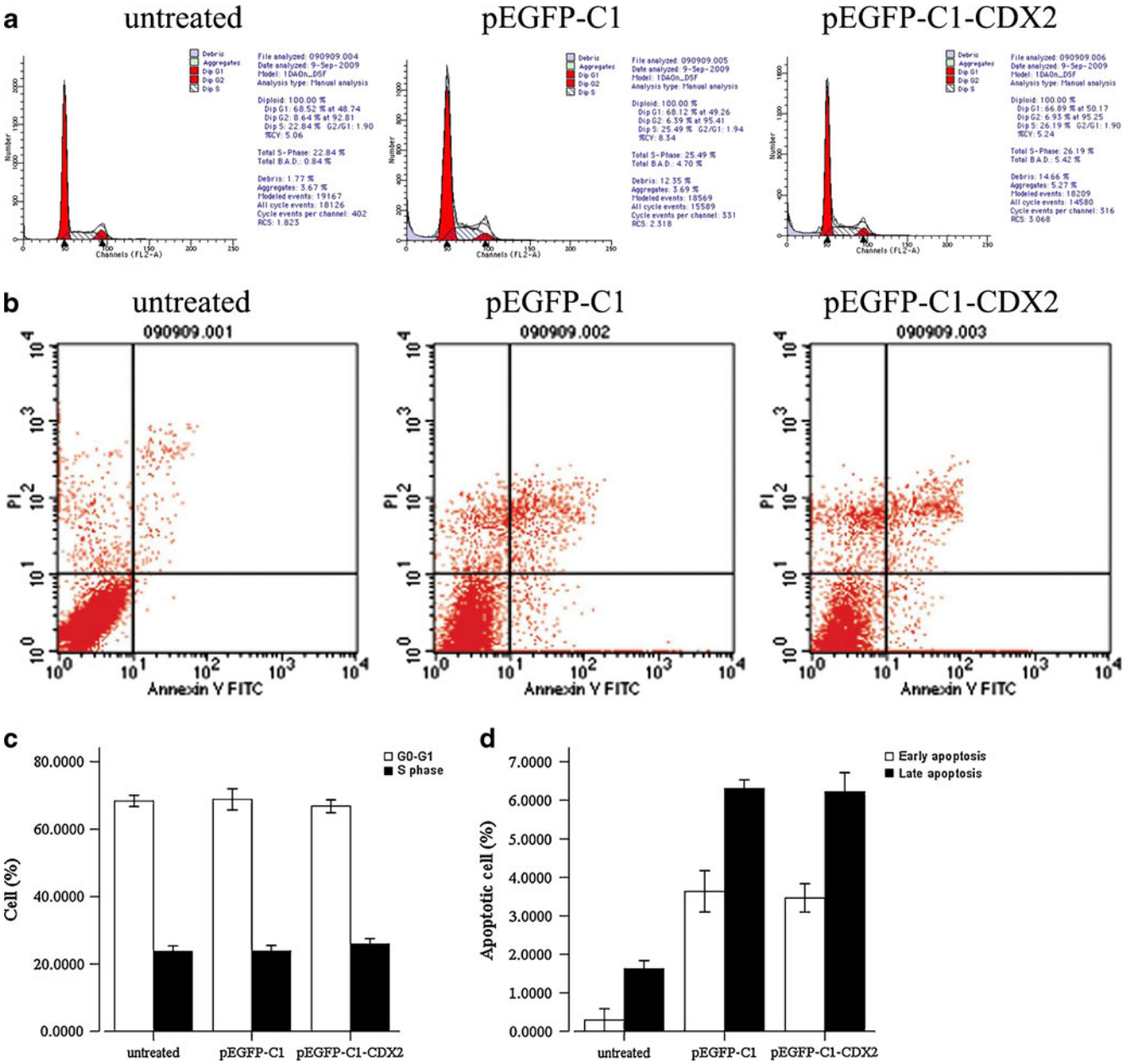

Fig. 3 Effects of CDX2 expression on cell cycle progression and apoptosis of Lovo cells. Cells were left untreated cells, or transfected with pEGFP-C1 or pEGFP-C1-CDX2. **a** and **c** Cells stained with PI were analyzed to determine cell cycle progression using flow cytometry. **b** and **d** Cells were incubated with FITC-conjugated Annexin V and PI 48 h after transfection. The percentage of apoptotic cells was determined using flow cytometry. The data are the mean ± SD mean of three independent experiments performed in duplicate

Effects of Exogenous CDX2 Expression on Lovo Cell Proliferation

Lovo cell proliferation was detected by the Methyl thiazolyl tetrazolium (MTT) assay after transient transfection, as described in the Materials and Methods. As shown in Fig. 2, there was no significant change in inhibition rates of pEGFP-C1-CDX2-transfected cells compared with pEGFP-C1-transfected cells ($P>0.05$).

Cell Cycle Progression and Apoptosis Analysis of CDX2-expressing Lovo Cells

Lovo cells transfected with pEGFP-C1 or pEGFP-C1-CDX2 were fixed, stained with PI, and the DNA content and cell cycle distribution measured using flow cytometry. Cell-cycle distribution analysis showed no significant change in the percentage of cells in G1- and S-phase in cells transfected with pEGFP-C1-CDX2 when compared with untreated and pEGFP-C1-transfected cells ($P>0.05$, Fig. 3a and c). Simultaneous detection of the apoptotic marker, Annexin-V, and propidium iodide incorporation revealed no significant differences in apoptosis (early- and late-phase) in CDX2-expressing cells compared with cells transfected with pEGFP-C1 ($P>0.05$, Fig. 3b and d). Of note, there was a slight increase in apoptosis in the pool of cells transfected with pEGFP-C1 or pEGFP-C1-CDX2 compared with untreated cells as a result of transfection (Fig. 3b).

Effect of Exogenous CDX2 Expression on MMP-2 Secretion in Lovo Cells

Activation of MMP-2 selectively degrades collagen type IV, contributing to tumor invasion and metastasis. MMP-2 activity is an important determinant of tumor cell invasive potential. Therefore we examined whether CDX2 regulates the secretion of MMP-2 from Lovo cells. The MMP-2 levels in the conditioned medium (CM) was determined by ELISA after transient transfection for various times (24 h, 48 h, 72 h), as described in the Materials and Methods. As shown in Fig. 4, MMP-2 levels were significantly decreased in the CM of cells transfected with pEGFP-C1-CDX2 compared to pEGFP-C1-transfected and untreated cells at 48 h, 72 h ($P<0.05$). MMP-2 levels in the CM of pEGFP-C1-transfected cells and untreated cells did not significantly change over time ($P>0.05$).

Expression of E-cadherin in CDX2-expressing Lovo Cells

To ascertain whether exogenous expression of CDX2 in Lovo cells mediates an epithelial-mesenchymal transition (EMT) phenotype, immunocytochemistry was performed to examine protein expression of the EMT marker, E-cadherin. Immunohistochemical analysis revealed that E-cadherin was found

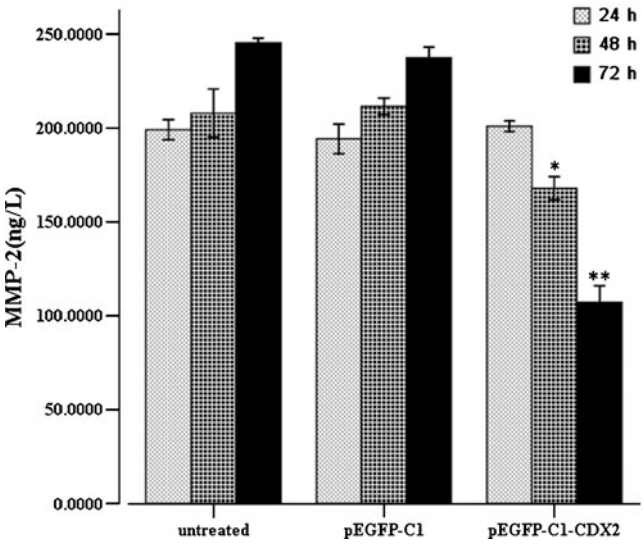

Fig. 4 CDX2 expression reduces MMP2 secretion. Levels of MMP-2 (ng/ml) in conditioned medium collected from untreated, pEGFP-C1-transfected, and pEGFP-C1-CDX2-transfected Lovo cells as determined at 24 h, 48 h, and 72 h. Data represent mean ± SD from three independent experiments. *$P<0.05$, **$P<0.01$

distributed in the cytomembrane and cytoplasm around the nucleus in untreated and pEGFP-C1-transfected Lovo cells (brown staining, Fig. 5a and b). An increase in E-cadherin expression was observed in Lovo cells transfected with pEGFP-C1-CDX2 (Fig. 5c), suggesting that CDX2 may regulate E-cadherin expression.

Effects of CDX2 Expression on the Invasive Potential of Lovo Cells

To observe the effect of CDX2 expression on the invasive potential of Lovo cells, pEGFP-C1-CDX2 and pEGFP-C1 were transfected into cells and stably expressing cell lines were established. As shown in Fig. 6, we assessed the effect of exogenous CDX2 expression on cell invasion. Representative photos show that invasion in pEGFP-C1-CDX2-transfected cells was markedly reduced compared with that of pEGFP-C1-transfected and untreated cells (Fig. 6a). Cells transfected with pEGFP-C1 and untreated cells show invasive activity in vitro ($P>0.05$). However, this invasive activity decreased significantly in cells that express exogenous CDX2 ($P<0.01$, Fig. 6b). These results demonstrate that a decrease in CDX2 expression is likely associated with the invasive potential of CRC.

Discussion

CDX2, a homeobox gene transcription factor family member, plays a critical role in the early differentiation,

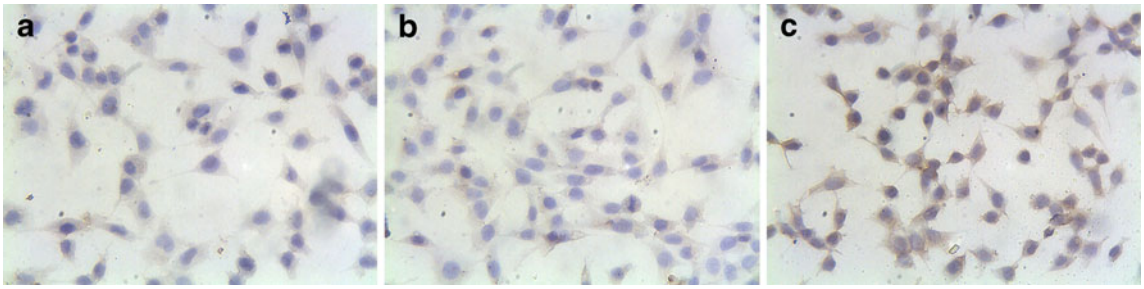

Fig. 5 Expression of E-cadherin in Lovo cells. Immunohistochemical analysis for E-cadherin expression in untreated (**a**), pEGFP-C1-transfected (**b**), and pEGFP-C1-CDX2-transfected (**c**) Lovo cells (×400). E-cadherin is located in cytomembrane and cytoplasm around the nucleus. Expression of E-cadherin increased in pEGFP-C1-CDX2-transfected Lovo cells

maintenance, and proliferation of intestinal epithelial cells. It has been suggested that *CDX2* is a master 'control gene' for intestinal epithelial differentiation and proliferation [14]. Previous observations have demonstrated that the CDX2 protein is likely a potent suppressor of tumorigenic growth and that CDX2 inactivation may contribute to the progression of CRC [21, 22]. In order to get further insight into the role CDX2 plays in colon carcinogenesis, particularly in tumor progression, we analyzed the consequences of exogenous CDX2 expression in Lovo cells.

Exogenous expression of CDX2 was reported to inhibit growth and induce apoptosis of the human colon cancer cell line, HT-29 [22]. However, we observed no significant differences in proliferation rate, cell cycle distribution, or apoptosis of pEGFP-C1-CDX2-transfected Lovo cells compared to pEGFP-C1-transfected cells. A previous report showed that Lovo cells have higher basal CDX2 expression levels compared to HT-29 cells [23]. Therefore, it is possible that HT-29 cells are more dependent on reduced CDX2 expression and that increases in its expression induce more dramatic effects on cell growth. Furthermore, CDX2 expression levels vary in human colorectal tumors [23]. Therefore, the differential responses in these two cells lines will be helpful in future studies to determine how

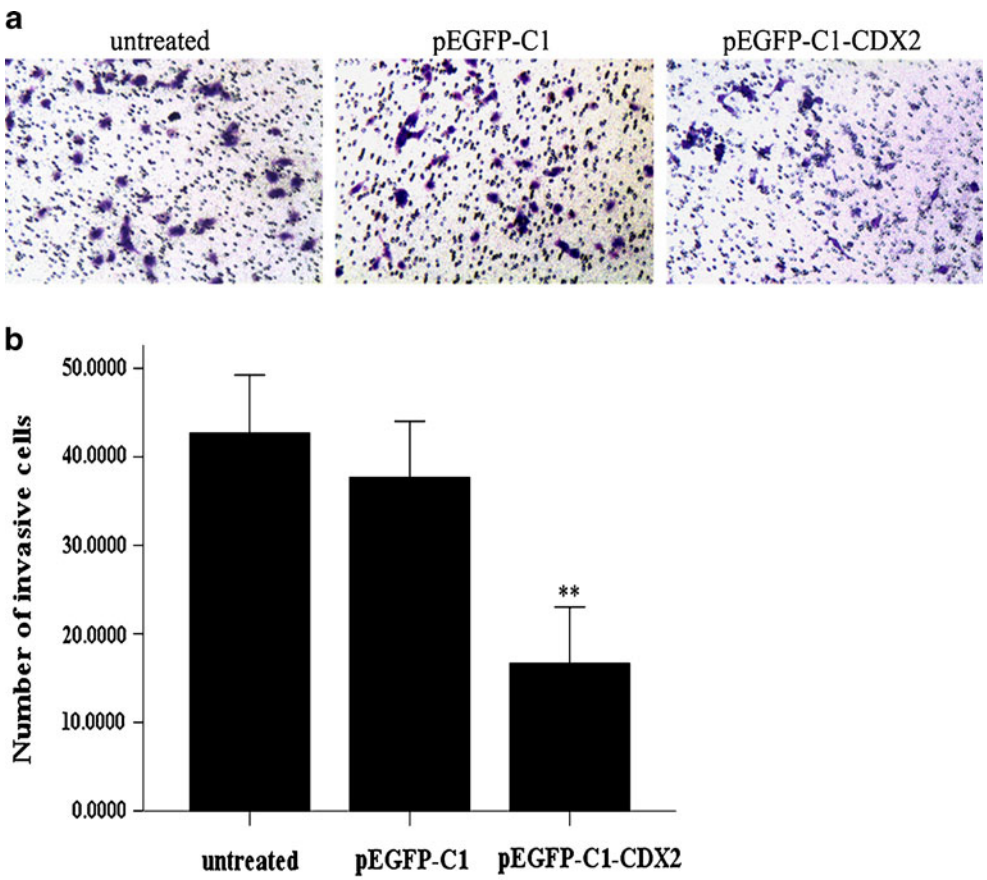

Fig. 6 Exogenous expression of CDX2 inhibits invasion of Lovo cells. **a** Representative photographs of invading Lovo cells (crystal violet staining, ×200 magnification). **b** Quantification of invading cell numbers. Each sample was assayed in triplicate. $^{**}P<0.01$

levels of CDX2 expression influence cell growth and to delineate pathways that may cooperate with reduced and/or complete loss of CDX2 expression.

As it had already been demonstrated that CDX2 expression levels decrease in invading human colon cancer cells, we investigated whether CDX2 may play a role in this process [21]. Invasion and metastatic potential requires proteolytic degradation of the extracellular matrix [24]. MMP-2 is thought to play an important role in tumor invasion and metastasis by selectively degrading type IV collagen [25, 26]. Based on this information, we hypothesized that CDX2 might regulate MMP-2 secretion. In our studies, we clearly show that exogenous expression of CDX2 in Lovo cells results in a significant decrease in MMP-2 secretion, which, subsequently, prevents cell invasion and migration in vitro. Therefore, it is plausible that CDX2 may serve as one of the major regulators of MMP-2 secretion in CRC.

EMT is a well characterized developmental process that has been considered to play a vital role in tumor progression [27, 28]. In primary tumors, the induction of EMT can lead to structural changes involving cell adhesion molecules, in particular E-cadherin. E-cadherin is a transmembrane glycoprotein that is localized to adherens junctions that are typically found in epithelial cells, and plays an important role in maintaining the structural integrity of epithelial sheets [29]. The loss of E-cadherin expression has been reported in several gastrointestinal (GI) cancers, including advanced colorectal carcinoma and gastric cancer [30, 31]. Funakoshi, et al. found that CDX2 expression in a colon cancer cell line, Colo 205, did not directly alter E-cadherin expression levels, but increased its trafficking to the cell membrane compartment [32]. This group subcloned murine CDX2 cDNA into the MIGR1 retroviral vector (murine stem cell virus (MSCV) promoter) and generated retrovirus to infect Colo 205 cells. However, our results demonstrate that expressing CDX2 from the pEGFP-C1 vector (human cytomegalovirus (CMV) promoter) increased the expression of E-cadherin protein in Lovo cells. It is likely that the use of different vectors results in differential CDX2 expression levels and likely contributes to these conflicting results between our group and the Lynch group. In agreement with our results, a recent study has shown that CDX2 levels are inversely correlated to Snail expression, a direct repressor of E-cadherin during EMT [33]. These results suggest that CDX2 expression may be negatively regulated by Snail during this process [34]. Therefore, we predict that increased expression of CDX2 in colon cancer cells would lead to increased E-cadherin expression and, ultimately, maintenance of the cell-cell adherens junction preventing tumor metastasis.

Finally, we further explored the implications of CDX2 expression on the invasive potential of Lovo cells, and established that exogenous CDX2 expression was associated with reduced cell invasion. These data suggest that CDX2 expression is physiologically significant for cell-cell adhesion and demonstrate that restoration of CDX2 expression might serve to limit the invasiveness and cell migration of colon cancer cells.

In conclusion, our results show that exogenous expression of CDX2 has negative consequences on malignant invasion and metastasis of colon carcinoma. Therefore, CDX2 might be considered as a therapeutic target for the treatment of colon carcinoma; exogenous expression of CDX2 in colon cancer cells in combination with chemotherapy may serve to reduce malignant progression of this disease.

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