Pancreatic duodenal homeobox-1 (PDX1) functions as a tumor suppressor in gastric cancer

Juan Ma1,2, Minhu Chen1, Jide Wang2, Harry H.X.Xia3, Senlin Zhu1, Yingjie Liang3, Qing Gu2, Liang Qiao2, Yun Dai2, Bing Zou2, Zesong Li2, Yusheng Zhang2, Huiyao Lan2 and Benjamin C.Y.Wong2,a

1Division of Gastroenterology and Hepatology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China, 2Department of Medicine, Queen Mary Hospital, University of Hong Kong, Hong Kong and 3Department of Pathology, The First Affiliated Hospital of Sun Yat-sen University, Guangzhou 510080, China

To whom correspondence should be addressed. Tel: +852 2855 5995; Fax: +852 2904 9443; Email: bcywong@hku.hk

Aim: Pancreatic duodenal homeobox-1 (PDX1) is a transcription factor of homeobox genes family important in differentiation and development of the pancreas, duodenum and antrum. This study aims to clarify the putative role of PDX1 in gastric carcinogenesis.

Methods: PDX1 expression was detected in gastric tissues with chronic gastritis and cancer as well as gastric cancer cell lines by immunohistochemistry, western blot, reverse transcription–polymerase chain reaction (RT–PCR) or quantitative real-time RT–PCR assays. The effects of PDX1 on cell proliferation, apoptosis, clone formation and migration were evaluated using cancer cell lines after transient or stable transfection with PDX1-expressing vector. The ability of PDX1 stable transfectant in tumor formation in xenograft mice was assessed. Results: PDX1 was strongly expressed in normal gastric glands, but was absent in 29 of 39 of human gastric cancer and most gastric cancer cell lines. Negative correlation between PDX1 and Ki-67 expression was found in both gastric tissues and cell lines. Ectopic overexpression of PDX1 significantly inhibited cell proliferation and induced apoptosis, accompanied by the activation of caspases 3, 8, 9 and 10. Overexpression of PDX1 also impaired the ability of cancer cells in clonal formation and migration in vitro. Furthermore, stable transfection with PDX1 reduced the ability of cancer cells in tumor formation in nude mice. Conclusions: PDX1 expression is lost in gastric cancers. Its effect on cell proliferation/apoptosis, migration and tumor formation in vitro and in vivo suggested that this protein functions as a putative tumor suppressor in gastric cancer.

Introduction

Homeobox genes that encode homeodomain transcription factors are involved in establishing gradients of differentiation during development of embryo and in maintaining these patterns in adult tissue (1–3). Aberrant regulations of homeobox gene expressions have been reported in various cancer or precancerous tissues including oral dysplasia and squamous cell carcinoma (4), acute lymphocytic leukemia (5,6), lung cancer (7), ovarian carcinoma (8) and hepatocellular carcinoma (9). Homeobox genes that are upregulated in cancers may be normally expressed during development and/or in undifferentiated cells, whereas the others that are downregulated in cancers may be normally expressed in adulthood and/or in differentiated tissues (10). Several homeobox proteins have been proposed to be positive or negative tumor modulators (7–11). For example, two caudal homeobox genes, CDX1 and CDX2, are normally limited in the intestine, and that the downregulation of CDX1 and CDX2 expression was found in colorectal cancers (10). CDX1 and CDX2 are frequently expressed in intestinal metaplasia and gastric carcinoma (12) and attribute to the development of intestinal differentiation along the gastric carcinogenesis pathway (13,14). However, the role of other homeobox genes in carcinogenesis of gastric cancer remains unclear.

The gene pancreatic duodenal homeobox-1 (PDX1), also known as insulin promoter factor-1, somatostatin transcription factor-1 or islet/duodenum homeobox-1, is located at chromosome 13q12.1 in the vicinity of the CDX2 gene. PDX1 protein belongs to a member of the ParaHox gene family transcription factors (15). PDX1 gene is first present in the dorsal cells of the gut at 8.5 days of the embryo and selectively expressed in adult endocrine glands such as pancreatic beta-cells, Brunner’s glands of the duodenum and pyloric endocrine cells of the stomach (16,17). This gene is critical for development of the pancreas and plays key role in regulating insulin gene in beta-cells of the endocrine pancreas (18). Recent studies reported that PDX1 is aberrantly expressed in pancreatic cancer (19–21).

PDX1 is found in three main endocrinal (gastrin, somatostatin and serotonin) cells of the distal stomach (22) and associated with gastric pathogenesis including autoimmune gastritis and pseudopyloric glands (23,24). In addition, PDX1-null mice embryos have malformations at the stomach–duodenum junction resulting in the lack of gastric empty and subsequent stomach distension (25). However, the function of PDX1 in gastric cancer is still unclear. PDX1 has been postulated to play roles in carcinogenesis of gastric cancer; however, no evidence has been obtained up to now.

In the current study, we aim to uncover the putative role of PDX1 in carcinogenesis of gastric cancer. By comparing the expression profile of PDX1 protein in cancer and paracancerous tissues and characterizing the role of this gene in regulating cancer cell growth both in vitro and in vivo, we report that PDX1 functions as a novel tumor suppressor in gastric cancer.

Materials and methods

Tissues specimens and cell lines

Formalin-fixed, paraffin-embedded specimens of 39 cases of gastric carcinomas surgically resected from patients were obtained from Department of Pathology, 37 pairs of fresh gastric cancerous tissues with paired non-cancerous tissues were collected from Division of Surgery and 13 cases of chronic gastritis biopsy specimens were collected from Division of Gastroenterology, The First Affiliated Hospital of Sun Yat-sen University, China. All tissue slices were subjected to histopathological review before the further investigation. The characteristics of patients and the histological features of the specimens were recorded and listed in Table I. All seven human gastric cancer cell lines have been described in our previous studies (26–29).

Immunohistochemistry

Paraffin-embedded tissue sections were incubated with the rabbit anti-PDX1 primary antibody (Santa Cruz Biotechnology, California, CA and Abcam, California, CA) (1:50) followed by incubation with peroxidase-conjugated anti-rabbit secondary antibody (Dako, Glostrup, Denmark) (1:100). The expression of PDX1 was then visualized using 1 mg/ml 3,3’-diaminobenzidine and counterstained with hematoxylin. PDX1 expression was quantitatively analyzed by a Leica DM2500 system microscope (Meyer Instruments, Houston, TX). The mean number of positive staining cells under four high powerful fields was counted. Positive rate was calculated according to the following equation: Y (cells per millimeter square) = X² (cells per high powerful field) × 0.0625/4 as suggested by Carl Zeiss Far East Company Limited (New Territories, Hong Kong).

Reverse transcription–polymerase chain reaction and quantitative real-time reverse transcription–polymerase chain reaction

After harvesting the cell, total RNA was extracted using Mini-REase RNA extraction kit (Qiagen, Hilden, Germany) and reversely transcribed to complementary DNA by Thermoscript RT system reagent (Gibco BRL, Gaithersburg MD) as per the manufacturer’s protocol. Polymerase chain reaction (PCR) was performed using 2 μl of complementary DNA and 0.2 U Hot start Taq DNA polymerase in 20 μl reaction system and for 30 cycle with 94°C denaturation for 30 s, 56°C annealing for 30 s and 72°C elongation for 45 s.
**Table I. Clinical characteristics and PDX1 protein expression of tissue specimens**

<table>
<thead>
<tr>
<th>Variable</th>
<th>Item</th>
<th>Chronic gastritis (n = 13)</th>
<th>Gastric cancer (n = 39)</th>
<th>P value</th>
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<tbody>
<tr>
<td>Age</td>
<td>Mean ± SE</td>
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<td>Female</td>
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<tr>
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<td>Antrum</td>
<td>7</td>
<td>12</td>
<td>0.332b</td>
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<td>4</td>
<td>19</td>
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<td>0</td>
<td>3</td>
<td></td>
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<tr>
<td></td>
<td>Cardia</td>
<td>1</td>
<td>3</td>
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<tr>
<td></td>
<td>Incisure</td>
<td>1</td>
<td>2</td>
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</tr>
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<td>PDX1c</td>
<td>Positive/negative</td>
<td>11/2</td>
<td>10/29</td>
<td>&lt;0.001b</td>
</tr>
</tbody>
</table>

*Pearson chi-square or Fisher’s exact test.

**Western blot analysis**

The whole cell lysates were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membranes and probed with first antibodies against PDX1, caspase 3, caspase 10 (Santa Cruz Biotechnology), Ki-67 (Abcam), caspase 8 (Alexis Biochemical, San Diego, CA) or caspase 9 (IMGENEX, San Diego, CA) followed by the horseradish peroxidase-conjugated secondary antibodies. Anti-glyceraldehyde-3-phosphate dehydrogenase antibody (Abcam) was used as an internal loading control. Antigen–antibody complexes were visualized by the enhanced chemiluminescence system (Amersham, Piscataway, NJ).

**Quantitative PCRs** were performed using Applied Biosystems Sequence Detection System 7900 (ABI Prism 7900HT, Applied Biosystems Company, Foster City, CA) with 10 μl mixture composed of Power SYBR GREEN PCR Master Mix (Applied Biosystems Company), 500 nmol of each primer and 300 ng of complementary DNA templates. The reactions were carried out with initial denaturation at 95°C for 5 min followed by 60 cycles of 20 s at 94°C, 20 s at 60°C and 40 s at 72°C. A final extension at 72°C for 5 min was included before a temperature ramp from 72°C to 95°C at 0.1°C/s with continuous fluorescent acquisition. The copy numbers of PDX1 messenger RNA (mRNA) were quantified by inspection of melt curves and CT values generated.

The primer sequences were as follows: reverse transcription–polymerase chain reaction (RT–PCR): PDX1, 5′-GGAGAAGAAAGGACCAATGG-3′ (forward) and 5′-GCCAGAGGAAAGGAGGACT-3′ (reverse), the proposed size of PCR product was 353 bp. Glyceraldehyde-3-phosphate dehydrogenase, 5′-GACCAACTGTTGCAATCC-3′ (forward) and 5′-GTGCCACCCCTGTGTCGTAA-3′ (reverse), the prospected size of PCR product was 454 bp. Real-time RT–PCR: PDX1, 5′-ATCTCCCCTACGAAGTGC-3′ (forward) and CGTGAGCTTTGGTGGATTTCAT (reverse), the prospected size of PCR product was 92 bp; glyceraldehyde-3-phosphate dehydrogenase, ATGGGGAAAGGTGTAAGGTC-3′ (forward) and GGGTGTAATGTGCAACAAAT (reverse) with 108 bp of PCR product. All PCR primers were designed online (http://frodo.wi.mit.edu/) and http://pga.mgh.harvard.edu/primerbank/.

**Real-time RT–PCR:** PDX1, 5′-(AATGGTTTCAATCAGGTTTCTG)T-3′ (forward) and 5′-(GATCTGCCCTTATGGTTTCAATCAGGTTTCTG)T-3′ (reverse), the prospected size of PCR product was 1328 bp.

**Cell growth assay**

Cells were seeded at 5.0 × 104 per well into 96-well plates. Cell proliferation was determined by measuring the absorption of cell proliferation reagent, WST-1 (Roche Molecular Biochemicals, Basel, Switzerland) as per the

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**Fig. 1.** PDX1 expression was weak or absent in gastric cancer cell lines comparing with matched normal tissues. (A and B) Protein and mRNA expression of PDX1 in gastric cancer cell lines were detected by western blot (A), RT–PCR (B) and real-time RT–PCR (C). Glycerinaldehyde-3-phosphate dehydrogenase (GAPDH) was used as the internal control for both assays. These experiments were repeated for three times with the same results. (D) PDX1 protein expression in chronic gastritis tissues, normal and metaplastic tissues adjacent to gastric cancer and cancer tissues was detected by immunohistochemistry. (E) mRNA expressions of PDX1 in paired gastric cancer and adjacent non-cancerous tissues were quantitatively detected by real-time RT–PCR. The ratios of mRNA level in cancers to non-cancerous counterparts were calculated. This experiment was repeated twice with identical finding.
manufacturer’s protocol. The ratio of absorbance at 480 nm wavelength of treated cells relative to that of untransfected cells was calculated and expressed as proliferation index. Each treatment was quadruplicated and the proliferation values were expressed as mean ± SEM.

Cell apoptosis assay
Cells were centrifuged, fixed and stained using Apo-Bromodeoxyuridine Apoptosis Detection Kit (BD Biosciences, San Jose, CA) and analyzed under fluorescent microscope according to the manufacturer’s protocol. The percentage of apoptotic cells in 1 × 10³ seeded cells was counted and expressed as apoptotic index. Each treatment was triplicated and the result was expressed as mean ± SEM.

Clone formation assay
Stable transfectants were seeded at 2 × 10³ per well into six-well plates and cultured for 14 days. Cell colonies were visualized under microscopy after staining with 0.005% crystal violet. Each treatment was set up in triplicate, and three independent experiments were performed.

Wound-healing assay
Stable transfectants were seeded at 1 × 10⁶ per well into six-well plates and grown to confluence for 24 h. The monolayer was wounded with a pipette tip and cells detached upon wounding were carefully rinsed off. The wound closure, as an index of migration, was defined as the percentage to the original wound width and observed over a 24 h period (0, 6, 12 and 24 h) and photographed under microscopy at 0 and 24 h.

Cell migration assay
Cell migration was assayed using CytoSelect 24-well cell migration assay kit (Cell Biolabs, San Diego, CA) according to the manufacturer’s protocol. The absorbance (optical density value) at 520 nm of wavelength of cell migration was measured. Each transfectant was assayed for three times and the result was expressed as mean ± SEM.

Tumorigenicity in nude mice
Cells (5 × 10⁶) in a 0.1 ml volume of phosphate-buffered saline were inoculated subcutaneously into the right flank of 15–20 μg weight female BALB/c-nu/nu mice (Laboratory Animal Unit, University of Hong Kong, Hong Kong) with four mice for each group. The Committee on the Use of Live Animals in teaching and research, University of Hong Kong approved the protocol. Tumor formation was observed 3 weeks later. The formed tumors were dissociated and freeze-dried for RNA extraction and their volumes were simply calculated as follows: \( V = \text{length} \times \text{width}^2 \).

Statistical analysis
Student’s t-test and by one-way analysis of variance with the Student–Newman–Keuls post hoc test were used to analyze difference in means of the continuous data. Pearson chi-square and Fisher’s exact test were used to analyze difference in ratios of the enumeration data. The Mann–Whitney U-test was used for non-parametric values. Correlation analyses were performed using Pearson linear correlation. All numeric data are expressed as mean ± SEM. A P value of <0.05 was considered statistically significant.

Results

PDX1 expression was weak or absent in gastric cancer cell lines
To investigate the relationship between PDX1 and gastric cancer, we first examined the expression of PDX1 in seven gastric cancer cell lines including N87, SGC7901, AGS, BCG823, KATOIII, MKN45 and SNU1. Normal human pyloric mucosa was used as control. As shown in Figure 1A, PDX1 protein with expected molecular weight of ~46 kDa was undetectable in most gastric cancer cell lines or only at extreme low level in N87 cells. This finding was confirmed by regular and real-time RT–PCR (Figure 1B and C). Normal pyloric mucosa expressed high level of PDX1 at both protein and mRNA levels. Our finding showed that PDX1 expression in gastric cancer was down-regulated.

PDX1 expression was downregulated in gastric cancer comparing with adjacent normal tissues
We next checked the expression profile of PDX1 protein in gastric cancer and non-cancerous tissues. Similar to the previous report regarding the nuclear and cytoplasmic localization of PDX1 in pancreatic metaplasia of the stomach (23), we also showed that PDX1-positive signal was highly presented in the nuclei and/or the cytoplasm of the epithelial cells in the glandular epithelium of chronic gastritis or normal tissues adjacent to gastric cancer (Figure 1D). On the contrary, low or undetectable PDX1 expressions were seen in gastric cancer tissues. Quantitative analysis revealed that the positive rate of PDX1 in chronic gastritis was 84.6% (11 of 13), whereas that in gastric cancer was only 25.6% (10 of 39). No significant difference was found between gastritis group and cancer group regarding to the background of patients such as age, gender as well as sampling sites (Table I).

In addition, we detected mRNA expression of PDX1 in paired gastric cancer and adjacent non-cancerous tissues by real-time RT–PCR. The ratios of PDX1 mRNA level in gastric cancers to non-cancerous counterparts were calculated. As shown in Figure 1E, the ratios in 31 of 37 pairs of tissues were <1. In consistent with protein profile, PDX1 mRNA expression was significantly decreased in gastric cancers compared with adjacent non-cancerous tissues (median, 0.0006 versus 0.0043, \( P = 0.021 \)). The mRNA findings were exactly
identical to protein patterns determined by immunohistochemistry of the correspondent tissues.

**PDX1 expression inversely correlated with Ki-67 expression**

We determined the expression of Ki-67, a commonly used marker for cell proliferation, together with PDX1 in gastric cancer by immunohistochemistry. We found that Ki-67 expression was gradually increased from adjacent normal tissues, adjacent metaplasia, to gastric cancerous tissues, whereas PDX1 expression was decreased simultaneously (Figure 2). Both quantitative analysis through counting the positive-stained cells under microscopy (Figure 2B, \( P \leq 0.001 \)) and Pearson linear correlation analysis showed a negative correlation between PDX1 and Ki-67 expression (correlation coefficient \( r = -0.771, P = 0.003 \)) (Figure 2C and D).

**Overexpression of PDX1 suppressed proliferation and induced apoptosis**

After transient transfection of pcDNA-PDX1 into SGC7901 cells, PDX1 expression was elevated markedly as compared with that of vector control (Figure 3A, top lane). Meanwhile, overexpression of PDX1 markedly decreased Ki-67 expression in SGC7901 cells (Figure 3A). Overexpression of PDX1 activated caspases 3, 8, 9 and 10 as evident by the increasing protein level of cleaved caspases (Figure 3A and B). In addition, the proliferation indices of SGC7901 cells transfected with pcDNA-PDX1 were decreased by 44.4\% at 48 h (\( P < 0.05 \)) (Figure 3C). The apoptotic indices of SGC7901 cells transfected with pcDNA-PDX1 increased by 80 and 50\%, respectively, 24 and 48 h after transfection, as compared with that of the vector control (\( P < 0.05 \)) (Figure 3D). Similar results were obtained in AGS cells (Figure 3C and D). When comparing with the vector control, the proliferation index of AGS-PDX1 cells was significantly decreased by 60.1\% 72 h posttransfection (\( P < 0.05 \)), whereas the apoptotic index in AGS-PDX1 cells increased by 90 and 25\%, respectively at 24 and 48 h posttransfection (\( P < 0.05 \)). These results suggested that PDX1 overexpression inhibited proliferation and induced apoptosis in gastric cancer. Similar results were found in AGS cells (data not shown).

**Overexpression of PDX1 suppressed the ability of clonal formation and cell migration in gastric cancer**

To confirm our *in vitro* findings using transient transfection, we established stable transfectants of PDX1 and vector control, pcDNA in SGC7901 cells. Three clones with increased expression of PDX1 identified by western blot were selected and termed as SGC-PDX1.5, SGC-PDX1.6 and SGC-PDX1.14 (Figure 4A). By using colony-forming assay, we found that SGC-PDX1 transfectant lost the colony-forming capacity by 68\% compared with vector control. The colonies formed by SGC-PDX1.6 and SGC-pcDNA were 82 ± 14 × 10\(^3\) versus 257 ± 67 × 10\(^3\) (\( P < 0.05 \), Figure 4B). The wound-healing assay showed that the wound closure in SGC-PDX1.6 cells was significantly slower than that of control at 24 h (\( P < 0.05 \), Figure 4C). The ability of cell migration in matrix gel in SGC-PDX1.6 was significantly lower than that of vector control (A520 value 0.060 ± 0.001 versus 0.274 ± 0.068, \( P < 0.05 \)) (Figure 4D). Based on these observations, we concluded that overexpression of PDX1 in gastric cancer cells suppressed its capacity of colony formation, wound healing as well as cell migration.

**Overexpression of PDX1 inhibited in vivo tumorigenesis of gastric cancer cells**

Finally, we asked whether the stable transfectants of PDX-1 could reduce tumorigenicity in nude mice xenografts. We found
significantly larger tumors in AGS-pcDNA transfectant with the average volume of 125.7 ± 16.3 mm³ compared with tumors of AGS-PDX1 cells with an average volume of 51.6 ± 19.5 mm³ (P, 0.05, Figure 5A). After detection of PDX1 expression in tumor resected from mice by RT–PCR, we found that the tumor formed by AGS-PDX1 expressed higher level of PDX1 than those formed by vector control (Figure 5B). Similar results were observed in SGC7901 transfectants (data not shown). These findings suggested that overexpression of PDX1 could inhibit tumorigenesis in vivo.

Discussion

In this study, we characterized the role of PDX1 in cell growth and tumorigenesis of gastric cancer. We showed that PDX1 expression was low or absent in most gastric cancerous tissues and cancer cell lines. Overexpression of PDX1 inhibited cancer cell growth and suppressed xenograft tumor formation in nude mice. Our finding showed that PDX1 is a novel tumor suppressor during gastric carcinogenesis.

In embryogenesis of mice, PDX1 is expressed in pancreas, duodenum and gastric pyloric glands, which was deformed in PDX1-deficient mice (16,17,22,30–32). Several groups reported that PDX1 was expressed in benign and malignant tumors derived from pancreas, breast, colon, prostate and kidney but not the adjacent normal tissues (19–21). However, there was no report to investigate PDX1 expression in gastric cancers as well as in different sites of healthy adult human stomach. Interestingly, we found that PDX1 was expressed not only by pyloric glands but also by corpus and fundus of patients with chronic gastritis but was absent in ~75% of tumors investigated. This finding was consistent with our previous report that gastric expression...
mice were resected for extraction of total RNA. PDX1 expression was downregulated in Helicobacter pylori-infected gastric mucosa and intestinal metaplasia compared with normal mucosa (33), suggesting that PDX1 was gradually lost during gastric carcinogenesis. We found that there were six cancer tissues expressed higher level of PDX1 than normal counterparts (Figure 1D), one may speculate if this different expressing profile was related to the histopathological type of gastric cancer. Our 37 samples included 28 intestinal type and two diffuse type of cancers. PDX1 expression seems not to be associated with histopathological type of gastric cancer. This finding is similar to Almeida’s report that CDX2 expression is not correlated to the histopathological type of gastric cancer (12). Nevertheless, further study remains to be performed by using more samples.

In addition, we showed that PDX1 expression in normal tissues was higher than that of gastric cancer cell lines. PDX1 expression in N87 was only slightly lower than that of normal control tissue. Taking into account that N87 was derived from a liver metastasis of a well-differentiated gastric carcinoma (http://www.atcc.org), our findings supported the previous conception that PDX1 expression was critical for cell differentiation in foregut organ or ectopic liver sites (34–36).

We have shown the important role of PDX1 in cell proliferation, apoptosis, clonal formation and migration capacity of gastric cancer cells in vitro. Finally, we tested the ability of PDX1 stable transfectant in forming tumors in vivo. When comparing with vector controls, stable transfectant of PDX1 formed smaller and slow growing tumors in xenograft nude mice. These findings suggested that PDX1 functioned as a tumor suppressor in gastric cancer. Because PDX1 was essentially a transcription factor, the downstream effecter of this protein remains to be identified in further study.

In summary, our study showed that PDX1 was generally expressed in normal glands of chronic gastritis and gradually lost in the cascade of gastric carcinogenesis. Similar to another homeobox gene, CDX2 gene that is a tumor suppressor in colon cancer (37), PDX1 also functioned as a tumor suppressor characterized by its ability to inhibit cancer cell proliferation, induce apoptosis, suppress cell migration and abrogate xenograft tumor formation in vivo. This serves as a potential therapeutic target for gastric cancer treatment in future.

**Fig. 5.** Overexpression of PDX1 inhibited in vivo tumorigenesis. (A) Stable transfectants (6 × 10^6) were inoculated subcutaneously into the right flank of BALB/c-nu/nu mice. Four mice were included for each treatment. Tumor volume was calculated after 3 weeks. V (mm^3) = length (mm) × width^2 (mm). *P < 0.05 comparing with AGS-pcDNA. (B) Tumors formed in nude mice were resected for extraction of total RNA. PDX1 expression was assayed by RT-PCR with glyceraldehyde-3-phosphate dehydrogenase as the internal control.

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