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Down-regulation of Pdc4 leads to epithelial to mesenchymal transition and promotes metastasis in mice

Qing Wang^{a,h}, Jiang Zhu^{a,b,h}, Yong Zhang^a, Zhenxiao Sun^c, Xiaoling Guo^d, Xin Wang^e, Eun Lee^f, Vasudevan Bakthavatchalu^a, Qifeng Yang^b, and Hsin-Sheng Yang^{a,g,*}

^aGraduate Center for Toxicology, College of Medicine, University of Kentucky, Lexington, KY 40536, USA

^bDepartment of Breast Surgery, Qilu Hospital, Shandong University, Jinan, Shandong Province, China

^cSchool of Chinese Pharmacy, Beijing University of Chinese Medicine, Beijing, P. R. China

^dDepartment of Oral and Maxillofacial Surgery, College of Dentistry, Shandong University, Jinan, Shandong Province, China

^eInstitute and Department of Urology, Peking University First Hospital, Beijing, China

^fPathology & Laboratory Medicine, University of Kentucky, Lexington, KY 40536, USA

^gMarkey Cancer Center, University of Kentucky, Lexington, KY 40536, USA

Abstract

In this study, we demonstrated that knockdown of Programmed cell death 4 (Pdc4), a novel tumor suppressor, decreased the expressions of epithelial-specific proteins and increased the expressions of mesenchymal-specific proteins *in vitro* and *in vivo*, suggesting that knockdown of Pdc4 results in epithelial to mesenchymal transition (EMT). Knockdown of Pdc4 increased the rate of wound closure and migration capacity in wound-healing assays and Boyden chamber migration assays, respectively, indicating that Pdc4 knockdown promotes cell migration. Pdc4 knockdown also altered the adhesion capacity of GEO cells to extracellular matrix including laminin, collagen IV, and fibronectin. To test whether knockdown of Pdc4 promotes metastasis *in vivo*, parental, control, and Pdc4 knockdown cells were injected into the cecal wall (orthotopic implantation) of nude mice. Tumors are formed on cecum in all injected mice. However, only mice injected with Pdc4 knockdown cells developed hepatic and local lymph node metastases. Immunohistochemical staining analyses showed that c-Myc and Snail/Slug expressions were up-regulated in the tumors from injection with Pdc4 knockdown cells. These results implicated that promotion of metastasis by Pdc4 knockdown was contributed by up-regulation of c-Myc and Snail/Slug in nude mice. Taken together, our data demonstrated that knockdown of Pdc4 leads to EMT, alternation of adhesion, and promotion of migration and metastasis.

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*Corresponding author: Address: Graduate Center for Toxicology, University of Kentucky, Lexington, KY 40536, USA. Tel: +1 859 323 6684; Fax: +1 859 323 1059, hyang3@uky.edu (H.S. Yang).

^hQing Wang and Jiang Zhu contributed equally to this work.

Conflict of interest Statement

There is no conflict of interest.

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Keywords

Pdcd4; EMT; migration; metastasis; orthotopic implantation; colon cancer

1. Introduction

Metastasis, the spread of tumor cells from a primary tumor site to a secondary site is the major cause of death in cancer patients. Detachment of tumor cells from primary tumor and invasion into surrounding normal tissues are important steps for tumor metastasis. During these steps, tumor cells require epithelial to mesenchymal transition (EMT) to lose cell-cell adhesion and gain motility.¹ EMT was first recognized as a conserved process during embryonic development. During EMT, cells acquire a fibroblastoid phenotype, lose epithelial cell polarity, down-regulate epithelial-specific proteins and induce various mesenchymal-specific proteins, and increase migration through extracellular matrix.² Loss of E-cadherin expression, an epithelial-specific protein, is a major indication of EMT, which is usually concomitant with the increase of mesenchymal N-cadherin expression, a mesenchymal-specific protein. This cadherin switch leads to loss of the affinity with epithelial neighbors and gain of the affinity for mesenchymal cells, resulting in increased migration and invasion.³ E-cadherin can be inactivated or silenced by various mechanisms including mutations and gene down-regulation through promoter hypermethylation and histone deacetylation. In addition, the zinc-finger transcription factors, Snail and Slug, have been reported to repress E-cadherin expression and increase tumor invasion in various malignancies.^{4, 5} Snail and Slug inhibit E-cadherin expression by binding to the proximal E-box of the E-cadherin promoter.⁴ Over-expression of Snail or Slug in epithelial cells has been shown to induce EMT and enhance invasion capacity.^{5, 6}

Programmed cell death 4 (Pdcd4), a novel tumor suppressor, is frequently down-regulated in several cancerous tissues compared to adjacent normal tissues including colon cancer tissue.⁷ Pdcd4 was first identified and its cDNA was cloned when cells were treated with apoptosis inducers.⁸ Over-expression of Pdcd4 has been shown to inhibit cell proliferation in the neuroendocrine Bon-1 cells,⁹ whereas knockdown of Pdcd4 promoted cell proliferation.¹⁰ Ectopic expression of *pdcd4* cDNA in mouse JB6 cells inhibits 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced transformation and tumor phenotype.^{11, 12} Conversely, down-regulation of Pdcd4 expression by *pdcd4* antisense resulted in an increase in TPA-induced transformation.¹³ In consistence with these observations, transgenic mice that over-expressing *pdcd4* cDNA in the skin showed significant reduction in 7,12-dimethylbenz(a)anthracene (DMBA)/TPA-induced skin papilloma formation and carcinoma incidence.¹⁴ Knockout of Pdcd4 in mice led to an increase in DMBA/TPA-induced papilloma.¹⁵ These findings suggest that Pdcd4 is able to inhibit the early stage of carcinogenesis.

In addition to inhibit tumor promotion, Pdcd4 has also been demonstrated to suppress tumor invasion. Ectopic expression of *pdcd4* cDNA suppressed invasion and intravasation in colon tumor RKO cell^{16, 17} and prostaglandin E2-induced invasion in breast tumor MCF7 cells,¹⁸ and ovarian tumor OVCA433 and SKOV3 cells.¹⁹ Knockdown of Pdcd4 expression promoted invasion in colon HT29 and GEO cells^{20, 21} as well as breast cancer MCF7 and D47T cells.²² Pdcd4 knockdown in colon tumor HT29 and GEO cells led to a fibroblast-like morphological change and promoted invasion.²⁰ Concurrently, Pdcd4 knockdown resulted in down-regulation of E-cadherin expression, translocation of β -catenin into nucleus, and activation of β -catenin dependent transcription.²⁰ Down-regulation of E-cadherin by Pdcd4 knockdown in colon HT29 cells was contributed, at least in part, by elevation of Snail expression since knockdown of Snail expression in the Pdcd4 knockdown cells reversed the

E-cadherin expression.²¹ The expression of c-Myc, the downstream targets of β -catenin dependent transcription, was found to be up-regulated by Pdc4 knockdown and knockdown of c-Myc inhibited invasion.²¹ Recently, we found that c-Myc stimulated MAP4K1 expression leading to activation of AP-1 dependent transcription in the Pdc4 knockdown cells.²³ Since AP-1 dependent transcription regulates several events required for cell invasion,²⁴ these findings suggest that c-Myc contributes to invasion induced by Pdc4 knockdown. Although knockdown of Pdc4 has been demonstrated to down-regulate E-cadherin expression and promote invasion in cell culture systems, it is unclear whether Pdc4 knockdown causes EMT and promotes metastasis *in vivo*.

In this study, we demonstrated that Pdc4 knockdown led to EMT, enhancement of cell migration, alternation of cell-matrix adhesion, and promotion of metastasis in nude mice. We also demonstrated that c-Myc and Snail/Slug expression were up-regulated in the primary tumors derived from injection of Pdc4 knockdown cells, revealing a mechanism that knockdown of Pdc4 promotes metastasis *in vivo*.

2. Materials and Methods

2.1. Cell lines and culture conditions

GEO cells were kindly provided by Dr. Douglas Boyd (MD Anderson Cancer Center) and HT29 cells were purchased from American Type Culture Collection (ATCC, Manassas, VA). GEO-shLacZ, GEO-shPdc4, HT29-shLacZ, and HT29-shPdc4 cells were generated as described previously.²⁰ All cells were grown in McCoy's medium containing 10% FBS, 2 mM L-glutamine, and 100U/ml penicillin-streptomycin and incubated at 37°C with 5% CO₂ in a humidified incubator.

2.2. Western blot analysis

Aliquots containing 20 or 40 μ g of protein were separated on a SDS-PAGE, and transferred to nitrocellulose membranes as described previously.¹¹ Subsequently, the membrane was incubated with antibodies against α -catenin (1:200 dilution, Santa Cruz Biotechnology, Santa Cruz), γ -catenin (1:200 dilution, Santa Cruz Biotechnology), N-cadherin (1:500 dilution, BD Biosciences, San Jose, CA), Fibronectin (1:500 dilution, BD Biosciences), or GAPDH (1:2000 dilution, Santa Cruz Biotechnology) followed by horseradish peroxidase-linked secondary antibody. The target protein was visualized by chemiluminescence (Denville Scientific, Metuchen, NJ).

2.3. Migration and wound-healing assays

For Boyden chamber migration assay, cells were incubated in McCoy medium containing 0.1% FBS for 24 h prior to migration assays. Cells (1×10^5 cells) were suspended in McCoy medium containing 0.1% BSA (McCoy-BSA) and seeded onto Transwell (8- μ m pore size, Corning, NY). EGF was diluted into McCoy-BSA at 20 ng/ml and added to the lower well. Chambers were incubated at 37°C for 24 h. The un-migrated cells on the upper surface of the filter were removed with cotton swabs. The filter then was fixed and stained with 0.1% (w/v) crystal violet in ethanol. The number of cells that traversed the filter into the lower compartment was determined by counting 7 random areas under Olympus CKX 41 microscope. For wound-healing assays, 5×10^4 cells were plated in each well in a 24-well plate. Once the cells reached 100% confluence, a "wound" was created using a sterile 200 μ l pipette tip. The wounded monolayer was then washed 3 times with complete medium to remove cell debris. Photographs of the wounded area were taken at the time 0 and 48 h. The width of the cell-free gap was measured by Olympus CKX41 microscope using Qcapture Pro software (QImaging, Surrey, BC, Canada). The percent of wound closure was determined by using the following formula: $[\Delta \text{ length}/\text{length (0 hour)}] \times 100\%$.

2.4. Cell adhesion assays

The adhesion assays were performed as follows. GEO-shLacZ or GEO-shPdcd4 cells were prepared in serum-free McCoy's medium, and 1×10^4 cells per well were seeded on 24-well plates coated with either Matrigel, collagen I, collagen IV, laminin, or fibronectin (1 μ g/well). Cells seeded on fibronectin were incubated at 37°C for 30 min while other cells were incubated for 15 min. After incubation, plates were washed twice with phosphate-buffered saline (PBS) to remove un-attached cells and stained with 0.1% (w/v) crystal violet in ethanol at room temperature for 20 min. After wash with distilled water three times, the stained cells were dissolved with 0.5% (v/v) Triton X-100. The optical density was measured at 595 nm and the cell numbers were estimated by using cell-number standard curve.

2.5. Orthotopic implantation of tumor cells

Five to six weeks old female Athymic nude (Hsd:Athymic Nude-Foxn1^{nu}) and SCID (ICRTaac:TCR-Prkdc<scid>) mice weighting 18 to 22 g were purchased from Harlan (Indianapolis, IN) and Taconic (Hudson, NY), respectively. Mice were maintained in the University of Kentucky facility under specific pathogen-free condition. Animal care procedures and experimental protocols were approved by the Institutional Animal Care and Use Committee based on guidelines from the NIH. All mice were fed with commercial diet, given water ad libitum, and subjected to a 12 h-light/12 h-dark cycle. The mice were anesthetized intraperitoneally with ketamine:xylazine cocktail (ketamine, 90mg/kg; xylazine, 10mg/kg). A small abdominal incision was made, and the cecum was exteriorized. Cells (2×10^6 cells) in 100 μ l of PBS were injected into the cecal wall from the serosal side using a 30-gauge needle on a 1-ml syringe. A visible bulla between the submucosal and subserosal tissues was formed. The cecum was returned to the abdominal cavity, and the peritoneum and skin were then closed with 4-0 Vicryl suture and metal clips, respectively. At 8-week post-injection, the lung, liver, lymph node, and cecum samples were collected.

2.6. Immunohistochemical (IHC) analyses

The tissue samples were fixed with 4% neutral buffered paraformaldehyde, embedded in paraffin, and sectioned into 4- μ m slices. Tissue sections were de-waxed by heating at 60°C, washed in xylene, and re-hydrated through a graded series of ethanol and water. After antigen retrieval, inactivation of endogenous peroxidase with 3% H₂O₂, and block of tissues with normal goat serum, the tissues were incubated with antibodies against Ki-67 (predilute, LifeTechnologies, Grand Island, NY), α -catenin (1:250 dilution, Abcam, Cambridge, MA), β -catenin (1:100 dilution, Cell Signaling), N-cadherin (1:300 dilution, Abcam), E-cadherin (1:400 dilution, Cell Signal Technology, Danvers, MA), c-Myc (1:100 dilution, Abcam), Snail/Slug (1:150 dilution, Abcam), and Pdcd4 (1:1500 dilution)²⁰ followed by biotin-linked secondary antibody using Histostain-Plus kit (LifeTechnologies). The tissue sections were visualized using 3,3'-diaminobenzidine (DAB) and counterstained with haematoxylin.

2.7. Statistical analysis

Differences in the migration, cell adhesion, and proliferation index between control (GEO-shLacZ) and Pdcd4 knockdown (GEO-shPdcd4) cells were analyzed using one-way ANOVA (<http://faculty.vassar.edu/lowry/anova1u.html>). Differences in the presence of tumor nodules in cecum, lymph node, liver, and lung between groups were compared using Fisher's exact test (<http://faculty.vassar.edu/lowry/tab2x2.html>). Differences were considered statistically significant at the $P < 0.05$ level. The statistical trend of liver metastasis with injecting HT29, HT29-shLacZ, and HT29-shPdcd4 cells was defined as P 0.1.

3. Results

3.1. Knockdown of Pdc4 leads to EMT

We demonstrated that knockdown of Pdc4 in colon HT29 and GEO cells resulted in a fibroblast-like transition and down-regulation of E-cadherin.²⁰ This finding suggests that knockdown of Pdc4 may lead to EMT. The expression switch from epithelial to mesenchymal marker genes is a hallmark of EMT. To analyze these changes induced by Pdc4 knockdown, the expression of epithelial and mesenchymal marker proteins in control (GEO-shLacZ and HT29-shLacZ) and Pdc4 knockdown (GEO-shPdc4 and HT29-shPdc4) cells were examined. The control and Pdc4 knockdown cells were generated by stable expression of *lacZ* shRNA and *pdc4* shRNA, respectively.²⁰ Knockdown of Pdc4 decreased the expression of epithelial marker proteins (α -catenin and γ -catenin) and concomitantly increased the protein level of mesenchymal marker protein (N-cadherin) in both GEO and HT29 cells (Figure 1A). Interestingly, knockdown of Pdc4 increased fibronectin expression in GEO cells but it was undetectable in the HT29 cells. To analyze whether the Pdc4 knockdown cells maintained the mesenchymal marker expression *in vivo*, the GEO-shLacZ and GEO-shPdc4 cells were injected into the cecum of nude mice. After 8 weeks, the tumors on cecum were collected, fixed with paraformaldehyde, sectioned, and stained with antibodies recognizing N-cadherin, β -catenin, E-cadherin, or α -catenin. The GEO-shLacZ derived tumors showed weak to moderate N-cadherin and β -catenin staining (mesenchymal markers, Figure 1B and D) but moderate to strong E-cadherin and α -catenin expressions (epithelial markers, Figure 1F and H). In contrast, GEO-shPdc4 derived tumors showed strong N-cadherin and β -catenin expressions (Figure 1C and E), while E-cadherin and α -catenin staining was not detectable (Figure 1G and I). The β -catenin expression in GEO-shPdc4 derived tumors was high in the nucleus (Figure 1E). In contrast, β -catenin was weakly expressed in the cytoplasm and nucleus in GEO-shLacZ derived tumors (Figure 1D). These findings suggested that the β -catenin dependent transcription was activated in the GEO-shPdc4 derived tumors. Interestingly, knockdown of Pdc4 in cultured GEO cells showed a decrease in β -catenin expression²¹ whereas the β -catenin expression was increased in the tumors derived from Pdc4 knockdown GEO cells (GEO-shPdc4). These Western blotting and IHC staining results indicate that knockdown of Pdc4 leads to the decrease in expression of epithelial proteins but increase in expression of mesenchymal proteins.

3.2. Knockdown of Pdc4 promotes cell migration

We have demonstrated that knockdown of Pdc4 promoted cell invasion.²⁰ Although promotion of cell invasion usually increases migration ability, some studies did show that increase in cell invasion has a little effect on cell motility.²⁵ Thus, it is necessary to find out whether knockdown of Pdc4 promotes cell migration. The cell motility of Pdc4 knockdown (GEO-shPdc4) cells and control (GEO-shLacZ) cells were measured with wound-healing assays and Boyden chamber migration assays. The rate of wound closure was used to measure cell mobility in wound healing assays. In a confluent monolayer cells, the ability of cells (adjacent to the scratch) to heal the “wound” was evaluated under a microscope. GEO-shPdc4 cells completed wound closure 100% within 48 h, but the control cells (GEO-shLacZ) only showed approximately 25% wound closure, indicating that the rate of wound closure was approximately 4-fold faster in GEO-shPdc4 cells than that in GEO-shLacZ cells (Figure 2A and B). In addition to wound healing assays, we also performed Boyden chamber migration assays to measure cell mobility. Control or Pdc4 knockdown cells were serum starved for 24 h and placed in the upper well of the transwell. The attractant (EGF, 20 ng/ml) was placed in the lower well. After 24 h, the unmigrated cells were removed and cells that had migrated to the lower face of the membrane were stained with 0.1% (w/v) crystal violet. As shown in Figure 2C, the migration ability of

GEO-shPdc4 cells was approximately 9-fold higher than that of GEO-shLacZ cells. These results suggested that knockdown of Pdc4 promoted cell migration in GEO cells.

3.3. Cell adhesion to extracellular matrices is altered by Pdc4 knockdown

Cell-matrix adhesions are essential for cell migration, tissue organization, and differentiation. For tumor invasion, tumor cells must be able to form new cell-matrix and cell-cell attachments, and break the existing ones. Since knockdown of Pdc4 enhances migration (Figure 2) and Matrigel invasion,²⁰ it is of interest to examine whether knockdown of Pdc4 alters cell-matrix interaction. GEO-shLacZ and GEO-shPdc4 cells were seeded on 24-well plates coated with various EMC, Matrigel, collagen I, collagen IV, laminin, or fibronectin. After incubation, each well was washed with PBS to remove the unattached cells. The number of cells attached to the well was determined. As shown in Figure 3, attachment to collagen IV or laminin-coated plates was significantly decreased in GEO-shPdc4 cells compared with GEO-shLacZ cells. In contrast, the adhesive capacity to fibronectin is about 2-fold higher in GEO-shPdc4 cells than that in GEO-shLacZ cells, revealing a functional link between fibronectin-mediated adhesion and Pdc4 knockdown-induced EMT. These results demonstrate that knockdown of Pdc4 alters cell-matrix adhesion.

3.4. Knockdown of Pdc4 promotes metastasis and tumor growth in mice

Previously, we have demonstrated that knockdown of Pdc4 promotes Matrigel invasion *in vitro*.²⁰ To provide *in vivo* evidence that knockdown of pdc4 promotes metastasis, cells (GEO, GEO-shLacZ, or GEO-shPdc4 cells) were injected into cecal wall of nude mice using orthotopic implantation method. The advantage of orthotopic implantation is that the cells are injected into a similar organ environment in which the colon cancer stages, dissemination patterns, and aggressiveness closely replicates all relevant metastatic sites observed in humans.²⁶ Eight weeks post-injection, samples of lung, liver, lymph node, and cecum were collected and the tumor nodules of each sample were determined. Tumors were formed in the cecum in the mice injected with GEO (six out of six), GEO-shLacZ (ten out of ten), or GEO-shPdc4 (eight out of eight) cells (Table 1). Local lymph node metastasis (Table 1) and hepatic metastasis (Figure 4A and Table 1) occurred in all mice injected with GEO-shPdc4 cells (eight out of eight). In contrast, no mice injected with GEO (parental) or GEO-shLacZ (control) cells showed hepatic or local lymph node metastasis (Table 1). In addition, none of the three injected cell types caused pulmonary metastasis (Table 1). We also injected HT29, HT29-shLacZ, and HT29-shPdc4 cells into cecal wall of SCID mice. Four out of five mice injected with HT29-shPdc4 showed liver metastasis. On the contrary, zero out of four mice injected with HT29 and one out of seven mice injected with HT29-shLacZ cells had liver metastasis (supplementary Table S1). These results indicate that Pdc4 knockdown in colon cells promotes their metastatic capacity in mice.

Interestingly, the size of GEO-shPdc4 derived tumor in the cecal wall is much bigger than GEO-shLacZ derived tumor (control) (Figure 4A). This observation suggests that knockdown of Pdc4 may increase cell proliferation *in vivo*. To confirm this, the tissues of both GEO-shLacZ and GEO-shPdc4 derived tumors were stained with Ki-76 antibody, a proliferation marker. The GEO-shPdc4 derived tumor tissues showed strong positive staining of Ki-67 in the nuclei while the GEO-shLacZ derived tumors showed weak nuclear staining (Figure 4B). The proliferation index of GEO-shPdc4 derived tumors was 45% whereas the proliferation index of GEO-shLacZ derived tumors were 13% (Figure 4C). The proliferation index was calculated as number of cells with positive Ki-67 staining per 500 cells counted $\times 100\%$. These results suggest that knockdown of Pdc4 promotes cell proliferation *in vivo*, which is in agreement with our previous findings that knockdown of Pdc4 increases cell proliferation in the HT29 cells.¹⁰

3.5. Histological observations

Histological examination of primary and metastatic tumors demonstrated a moderately differentiated adenocarcinoma in the cecum when GEO-shLacZ cells were injected (Figure 5A). In contrast, the GEO-shPdc4 cell derived tumors formed a poorly differentiated adenocarcinoma in the cecum (Figure 5B). Several large tumors from GEO-shPdc4 cells injection were found to have central necrosis. As in the primary cecal tumors from the GEO-shPdc4 injection, the metastatic tumors in the liver were poorly differentiated (Figure 5C).

3.6. Knockdown of Pdc4 up-regulates c-Myc and Snail/Slug expression in mice

In the previous studies, we have demonstrated that knockdown of Pdc4 up-regulated c-Myc and Snail expression in the cultured cells.²¹ c-Myc is a transcription factor that activates genes involved in cell growth and represses genes involved in anti-proliferation and anti-metastasis.²⁷ Snail is a transcription repressor, which inhibits E-cadherin expression. Over-expression of Snail in epithelial cells promotes cell invasion.⁵ To investigate whether Pdc4 knockdown up-regulates c-Myc and Snail expression *in vivo*, IHC staining of tissue slides from GEO-shLacZ and GEO-shPdc4 derived tumors using antibodies against Pdc4, c-Myc, or Snail/Slug (antibody recognizes both Snail and Slug) were performed. As expected, GEO-shLacZ derived tumors showed a strong, cytoplasmic and nuclear Pdc4 staining (Figure 5D) while GEO-shPdc4 derived tumors had no or very weak staining of Pdc4 (Figure 5E). The GEO-shLacZ derived tumors were weakly stained for c-Myc (Figure 5F) whereas GEO-shPdc4 derived tumors were moderately to strongly stained for c-Myc in the nuclei (Figure 5G). The GEO-shLacZ derived tumors only showed a weak Snail/Slug expression (Figure 5H) but the Snail/Slug staining was strong in the nuclei in the GEO-shPdc4 derived tumors (Figure I). These results indicate that Pdc4 knockdown up-regulates c-Myc and Snail/Slug expression *in vivo*.

4. Discussion

In this study, we demonstrated that knockdown of Pdc4 decreased expression of epithelial proteins (α -catenin and γ -catenin) and increased expression of mesenchymal proteins (N-cadherin and fibronectin) in cultured cells (Figure 1A). In addition, the GEO-shPdc4 derived tumors showed a lower E-cadherin and α -catenin expressions and a higher N-cadherin and β -catenin expressions comparing to GEO-shLacZ derived tumors. In combination with our previous *in vitro* findings,²⁰ these results suggest that knockdown of Pdc4 leads to EMT. This conclusion is further supported by the findings that knockdown of Pdc4 promotes migration (Figure 2), decreases collagen IV and laminin adhesion, and increases fibronectin adhesion (Figure 3) because EMT usually increases cell motility and alters cell-matrix adhesion.² Since fibronectin mainly interacts with integrins,²⁸ the finding of increased adhesion with fibronectin by Pdc4 knockdown (Figure 3) suggests that integrins might be up-regulated in the Pdc4 knockdown cells. Integrins are heterodimeric transmembrane proteins consisting of α and β subunits. There are 18 known α subunits and 8 β subunits that combine into 25 different integrins. Which integrin components and how integrins are up-regulated by Pdc4 knockdown remain unclear.

The finding that the GEO-shPdc4 derived tumors display a lower level of E-cadherin than GEO-shLacZ derived tumors (Figure 1) suggests a weak cell-cell interaction in the Pdc4 knockdown cells *in vivo*. E-cadherin is a transmembrane glycoprotein on the cell surface, which plays a major role in cell-cell interaction in epithelial cells. The extracellular domain of E-cadherin mediates hemophilic, calcium-dependent interactions required for adhesion, whereas the intracellular domain interacts with the actin cytoskeleton through α -catenin, p120, and β -catenin.²⁹ Loss of E-cadherin expression is frequently found during tumor progression in most epithelial cancers. Immunohistochemical staining of human cancer

tissues showed that the level of E-cadherin expression often is inversely correlated with the tumor grade and stage.^{30, 31} Over-expression of E-cadherin results in reversion from invasive to benign tumor phenotype in cultured tumor cells.³² Reduced E-cadherin expression in colon tumor cells correlates with the increased invasive capacity.³³ Using *Rip1Tag2* mice of pancreatic β -cell carcinogenesis, Perl *et al.* demonstrated that the loss of E-cadherin mediated cell-cell adhesion is causally involved in the progression from adenoma to carcinoma.³⁴ Thus, E-cadherin functions as an invasion suppressor.

How does knockdown of Pdc4 promote invasion and/or metastasis? One possible mechanism is through activation of β -catenin dependent transcription. Given that E-cadherin is the binding partner of β -catenin, a decrease in E-cadherin expression results in an increase of free β -catenin in cytoplasm. The free β -catenin is then rapidly phosphorylated by glycogen synthase kinase 3 β (GSK3 β) in the adenomatous polyposis coli (APC)-axin-GSK3 β -casein kinase I complex, and subsequently degraded by the proteasome pathway. If the APC is mutated or GSK3 β activity is blocked by Wnt signaling, β -catenin will not be degraded and will translocate into nucleus leading to the activation of β -catenin dependent transcription.³⁵ Several target genes of β -catenin dependent transcription have been found to involve in the tumor invasion and metastasis.³⁶ Hence, down-regulation of E-cadherin is believed to promote invasion or metastasis through activation of β -catenin dependent transcription. Our IHC staining data showed that GEO-shPdc4 derived tumors displayed very weak expression of E-cadherin and strong accumulation of β -catenin in the nucleus (Figure 1), suggesting that promotion of metastasis by Pdc4 knockdown is through activation of β -catenin dependent transcription. In addition, c-Myc, a downstream target of β -catenin dependent transcription, showed a stronger staining in the GEO-shPdc4 derived tumors than in GEO-shLacZ derived tumors (Figure 5), indicating that c-Myc expression was up-regulated in the GEO-shPdc4 derived tumors. Recently, c-Myc has been demonstrated to activate JNK signaling pathway and AP-1 dependent transcription in Pdc4 knockdown cells.²³ Since AP-1 regulates several required events for tumor migration and invasion,²⁴ activation of AP-1 dependent transcription by c-Myc suggests that c-Myc regulates invasion/metastasis in the Pdc4 knockdown cells. This argument is further supported by the finding that knockdown of c-Myc in the GEO-shPdc4 cells inhibits invasive capacity.²¹ These findings reveal that Pdc4 knockdown promotes metastasis, at least in part, through up-regulation of c-Myc expression *in vivo*.

Previously, we demonstrated that knockdown of Snail in GEO-shPdc4 cells activates E-cadherin promoter activity.²¹ In addition to the *in vitro* data, here, we found that Snail/Slug expression is inversely correlated with E-cadherin expression in the GEO-shPdc4 derived tumors (Figure 1 and 5), suggesting that Snail regulates E-cadherin expression *in vivo*. Snail is a key transcription repressor for regulating E-cadherin expression. Over-expression of Snail in epithelial cells promotes invasion.⁵ Conversely, knockdown of Snail up-regulates E-cadherin expression and inhibits invasion.³⁷ Recent studies by Evdokimova *et al.* showed that the 5' untranslated region (5' UTR) of *snail* mRNA forms a stable secondary structure and translation of *snail* mRNA is regulated by Y-box binding protein 1 (YB-1), suggesting that protein translational control contributes to the regulation of Snail expression.³⁸ Pdc4 is a translation inhibitor that preferentially inhibits translation of mRNA with secondary structure at 5' UTR.³⁹ Therefore, Pdc4 may directly inhibit translation of Snail. Alternatively, Pdc4 may directly interact with helix-loop-helix domain of Twist1 to inhibit YB-1 expression resulting in suppressing Snail expression.⁴⁰ In addition, knockdown of Pdc4 has been recently demonstrated to activate AKT kinase activity and increase the phosphorylation of 4E binding protein (4EBP) and translation initiation factor (eIF) 4B, two AKT downstream targets.¹⁰ Hyperphosphorylation of 4EBP dissociates the cap binding protein eIF4E and results in the activation of cap-dependent translation.⁴¹ Increased phosphorylation of eIF4B has been shown to enhance translation and neoplastic

transformation.⁴² Thus, it is possible that Pdcd4 regulates Snail translation through activation of eIF4E and eIF4B. Whether Pdcd4 regulates Snail translation needs further investigation.

Taken together, the results in this study demonstrate that knockdown of Pdcd4 promotes migration and metastasis. In addition, over-expression of Pdcd4 inhibits tumor cell invasion.^{16–18} Thus, Pdcd4 might function as a suppressor of tumor invasion and metastasis. Agents for elevating Pdcd4 expression might be a promising therapeutic strategy in cancer treatment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A

Fig. 1. Knockdown of Pdc4 down-regulates epithelial proteins and up-regulates mesenchymal proteins. (A) Expression of epithelial proteins: α -catenin and γ -catenin, mesenchymal proteins: N-cadherin and fibronectin, and GAPDH were examined in control (GEO-shLacZ and HT29-shLacZ) and Pdc4 knockdown (GEO-shPdc4 and HT29-shPdc4) cells using Western blotting analysis. (B–I) GEO-shLacZ and GEO-shPdc4 cells were orthotopically injected into mice as described in Materials and Methods. After 8 weeks, the tumor nodules in the cecum were collected, fixed, and embedded. Paraffin sections from GEO-shLacZ (B, D, F, H) and GEO-shPdc4 (C, E, G, I) derived tumors were stained using IHC with antibodies against N-cadherin (B, C), β -catenin (D, E), E-cadherin (F, G), and α -catenin (H, I). Scale bar: 50 μ m.

A **B**

C **Fig. 2.**

Knockdown of Pdc4 promotes cell migration. (A) Wound-healing assays. GEO-shLacZ and GEO-shPdc4 cells were seeded onto a 24-well plate. At 100% confluence, cell monolayer was scratched with a plastic tip. At 0 h and 48 h after the scratch, the distance of wounded monolayer was measured under a microscope equipped with a digital camera. The representative images are shown. (B) The percentage of wound closure. Each value is expressed as the mean $\pm$ standard deviation (SD) of three independent experiments. The asterisk indicates significant difference compared with GEO-shLacZ cells as determined by one-way ANOVA ($P < 0.01$). (C) Boyden chamber migration assays. The number of cells that has migrated to the lower side of membrane surface was determined by counting 7 areas. The migration capacity of GEO-shLacZ cells is designed as 1. Two independent experiments were performed with triplicates for each sample. The representative data are

shown and expressed as the mean $\pm$ SD. The asterisk indicates a significant difference compared with GEO-shLacZ cells as determined by one-way ANOVA ($P<0.05$).

Fig. 3.

Pdc4 knockdown alters the adhesive capacity to ECM components. The cells attached to the ECM component were determined as described in Materials and Methods. The number of adhered GEO-shLacZ cells is designated as 100%. Two independent experiments were performed with 5 replicates for each sample. The representative data are shown and expressed as the mean $\pm$ SD. The asterisk indicates a significant difference compared with GEO-shLacZ cells as determined by one-way ANOVA ($P < 0.05$).

A **B**

Fig. 4. Knockdown of Pdc4 promotes metastasis and proliferation in nude mice. GEO-shLacZ and GEO-shPdc4 cells were orthotopically injected into mice as described in Materials and Methods. After 8 weeks, the tumor nodules in the cecum and liver were collected, fixed, and embedded. (A) The representative tumor nodules in cecum and liver are shown. Arrows indicate the tumor nodules. (B) Paraffin sections from GEO-shLacZ and GEO-shPdc4 derived tumors were stained with Ki-67 antibody. The representative images are shown. The brown color indicates the positive staining of Ki-67 in the nuclei. (C) The proliferation index. The proliferation index was calculated as number of Ki-67 positive staining cells per 500 cells counted. Two independent tumors were chosen with counting 3 areas each. The representative data are shown and expressed as the mean $\pm$ SD. The asterisk indicates significant difference compared with GEO-shLacZ cells as determined by one-way ANOVA ($p < 0.0005$).

Fig. 5. Histological and IHC analyses of GEO-shLacZ and GEO-shPdc4 derived tumors. (A-C) Histological analyses of tumor samples. Paraffin sections from GEO-shLacZ derived colon tumor (A), GEO-shPdc4 derived colon tumor (B), and GEO-shPdc4 derived metastatic liver tumor (C) were stained by haematoxylin and eosin. The representative images are shown. T: tumor tissue, N: normal tissue, Nec: Necrosis. Scale bar: 100 μ m. (D-I) IHC analyses of tumor samples. Paraffin sections from GEO-shLacZ (D, F, H) and GEO-shPdc4 (E, G, I) derived tumor were stained by IHC with antibodies against Pdc4 (D, E), c-Myc (F, G), and Snail/Slug (H, I). Representative images are shown. Scale bar: 50 μ m.

Table 1

Tumorigenicity and metastatic ability of GEO, GEO-shLacZ, and GEO-shPdcd4 cells in nude mice.

Cell line	Tumorigenicity	Regional lymph node metastasis	Liver metastasis	Lung metastasis
GEO	6/6	0/6	0/6	0/6
GEO-shLacZ	10/10	0/10	0/10	0/10
GEO-shPdcd4	8/8	8/8 ^{a,b}	8/8 ^{a,b}	0/8

Values are number of positive mice/number of injected mice

^a $P = 0.00033$ as compared with GEO cells

^b $P = 0.00002$ as compared with GEO-shLacZ cells