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Original Article

Impact of prospero homeobox-1 on tumor cell behavior and prognosis in colorectal cancer

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Abstract: Prospero homeobox 1 (PROX1) is up-regulated in colorectal cancer and plays an oncogenic role. In the present study, we sought to investigate the impact of PROX1 on oncogenic processes and to assess the prognostic value of PROX1 expression in colorectal cancer. A small interfering RNA or pcDNA6-myc vector was used to control *PROX1* gene expression in colorectal cancer DLD1 and SW480 cell lines. The expression of PROX1 in colorectal cancer tissues was investigated by immunohistochemistry. Angiogenesis, lymphangiogenesis, and tumor cell proliferation were assessed by analyzing the expression of respective markers of these phenomena, CD34, D2-40, and Ki-67 after immunohistochemical staining. PROX1 knockdown decreased both umbilical vein endothelial cell invasion and tube formation, down-regulated the expression of VEGF-A and HIF-1α, and up-regulated the expression of angiostatin. Lymphatic endothelial cell invasion and tube formation as well as the expression of VEGF-C were also suppressed by PROX1 knockdown. PROX1 knockdown suppressed tumor cell proliferation, migration, invasion, and epithelial-mesenchymal transition. In contrast, PROX1 overexpression enhanced tumor cell angiogenesis, lymphangiogenesis, proliferation, migration, invasion, and epithelial-mesenchymal transition. Levels of phosphorylated Akt, GSK3β, and MAPK were decreased by PROX1 knockdown and increased by PROX1 overexpression. PROX1 expression positively correlated with tumor size, extent of differentiation, lymphovascular invasion, depth of invasion, lymph node metastasis, stage, and poor survival. The mean microvessel density and Ki-67 labeling index values of PROX1-positive tumors were significantly higher than those of PROX1-negative tumors. However, there was no significant correlation between PROX1 expression and lymphatic vessel density. These results indicate that PROX1 influences tumor progression in colorectal cancer by regulating angiogenesis and tumor cell proliferation.

Keywords: Prospero homeobox 1, proliferation, angiogenesis, prognosis, colorectal neoplasm

Introduction

Colorectal cancer still remains one of the most common causes of cancer-related deaths despite notable improvements in patient survival achieved in recent decades [1-3]. Metastasis is directly or indirectly responsible for the majority of cancer-related deaths [4, 5]. Thus, it is very important to elucidate mechanisms underlying metastasis in order to develop effective therapeutic strategies for colorectal cancer [6].

Metastasis of tumor cells to secondary sites via blood and lymphatic vessels represents a common step in tumor invasion. Dysregulation of angiogenesis and lymphangiogenesis is functionally important in carcinogenesis and cancer progression [7-9]. Furthermore, it has been demonstrated that epithelial-mesenchymal transition (EMT) in various cancers is a crucial step leading to tumor metastasis [10-12]. Thus, investigations of these phenomena and other aspects of molecular and cellular biology of cancer should provide insights into the mechanisms of tumor metastasis and facilitate the development of therapeutic strategies to re-strict metastatic spread.

Prospero homeobox 1 (PROX1) is a homeobox-containing transcription factor related to the *Drosophila prospero* gene, which regulates cell fate and development of various organs including central nervous system, lens, liver, retina, heart, pancreas and lymphatic system [13]. It has been established recently that PROX1 has a variety of roles and its functions may change

according to the type of cancer [13]. On one hand, PROX1 acts as a tumor suppressor in hepatocellular carcinoma, esophageal cancer, pancreatic cancer, oral cancer, hematologic malignancy, sporadic breast cancer, and carcinoma of the biliary system [14-20]. At the same time, PROX1 promotes aggressive behavior of colorectal cancer, kaposiform hemangioendothelioma, and glioma. These latter observations point to a distinct oncogenic role of this protein [21-24].

Recently, PROX1 has been associated with neoplastic transformation, tumor differentiation, and poor prognosis in colorectal cancer [25-28]. In addition, PROX1 knockdown strongly suppressed EMT, whereas PROX1 overexpression greatly promoted it [25]. These results suggest that PROX1 is involved in colorectal carcinogenesis and thus, may be a candidate oncogene for colorectal cancer treatment. Therefore, to optimize treatment of colorectal cancer, it may be useful to elucidate the mechanism by which PROX1 promotes tumor progression. This is also important because the role of PROX1 in tumor angiogenesis and lymphangiogenesis in colorectal cancer still remains unclear.

The aims of the present study were to investigate the impact of PROX1 on invasive phenotypes of colorectal cancer cells and to examine its prognostic significance in patients with colorectal cancer.

Materials and methods

Cell culture and siRNA transfection

Human colorectal cancer cell lines DLD1 and SW480 were obtained from the American Type Culture Collection (Manassa, VA, USA). Cells were cultured in the Dulbecco's Modified Eagle's medium (DMEM) (Hyclon, Loan, UT, USA) supplemented with 10% fetal bovine serum and antibiotics. PROX1 small interfering RNA (siRNA) and scramble siRNA were purchased from Bioneer (Daejeon, Korea) and Qiagen (MD, USA), respectively. PROX1 cDNA was subcloned into pcDNA6-myc vector (Invitrogen, Carlsbad, CA, USA). PROX1 construction was verified by sequencing. The specific genes were transfected using lipofectamine™ RNAiMAX and lipofectamine™ 2000 (Invitrogen) according to the manufacturer's recommendations. Stable transfectant with empty-pcDNA 6-myc vector and pcDNA 6-myc-PROX1 was isolated by selection with 10 μg/ml blasticidin (Invitrogen) for 4 week and maintained with DMEM medium (Hyclon) supplemented with 10 μg/ml blasticidin (Invitrogen). To obtain the conditioned medium (CM), gene transfectecd cells were incubated in serum free medium for 1 day. Human umbilical vein endothelial cells (HUVECs) and human lymphatic endothelial cells (HLECs) were purchased from Lonza (Walkersville, MD, USA) and ScienCell (San-Diego, CA, USA), respectively. HUVECs and HLECs were maintained in the EBM™-2 medium supplemented with EGM™-2 Single Quotes™ kit (Lonza).

Proliferation assay

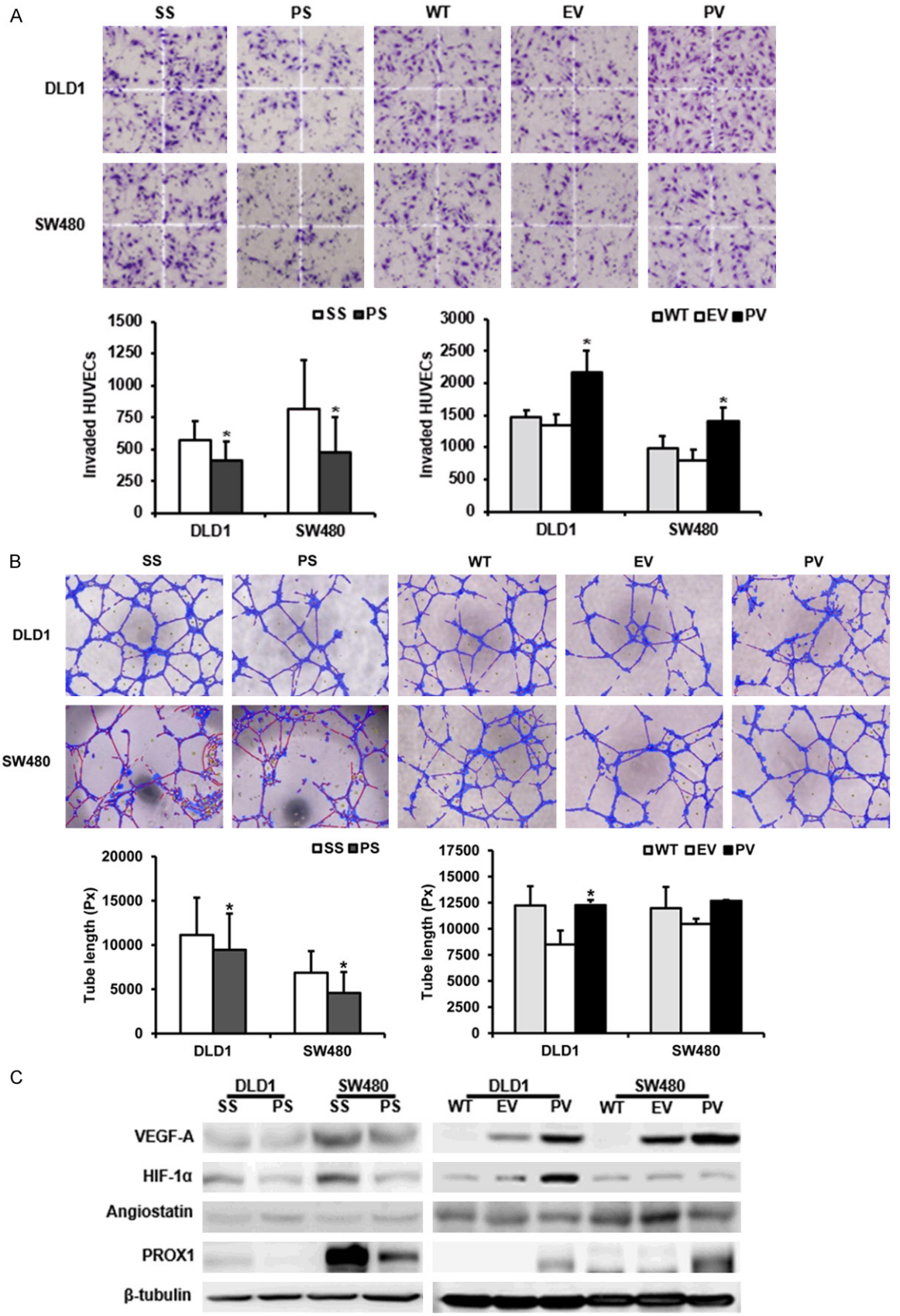
The water-soluble tetrazolium salt reagent (WST-1) (Daeil Lab Inc., Seoul, Korea) was used to measure proliferation of transfected cells. Transfected DLD1 and SW480 cells were seeded at a density of 1×10^4 cells/well in 96-well plates. After overnight incubation, the cells were treated with WST-1 reagent for 1 h at 37°C. Optical density was measured at 450 nm with a microplate reader (Infinite M200; Tecan Austria GmbH, Austria).

Matrigel invasion assay

To analyze the invasion of endothelial cells, HUVECs and HLECs were resuspended in 120 μL EGM®-2 MV Single Quotes® media and inoculated into transwell upper chambers (8-μm pores, Corning Inc., NY, USA) coated with matrigel (BD Bioscience, San Jose, CA, USA). Lower chamber was filled with prepared CM. After incubation for 3 h, invaded cells on the bottom surface of the transwell were fixed with 70% ethanol and stained with Diff-Quik solution (Sysmex, Kobe, Japan). Cells remaining on the top of upper chambers were wiped off with a cotton swab. Stained cells in lower chambers were counted in 5 selected fields under a light microscope.

In vitro endothelial tube formation assay

Geltrex™ reduced growth factor basement membrane matrix (Invitrogen) was used for *in vitro* endothelial tube formation assays. Ninety-six well plates were coated with Geltrex™ matrix, which was allowed to polymerize at 37°C. HUVECs and HLECs were suspended in CM and added to the top of the Geltrex™ matrix. Cells were incubated at 37°C in the atmosphere of 5% CO_2 overnight and photographed


A
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WT EV PV
3000
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1500
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B
Tube length (Px)
20000
15000
10000
5000
0
17500
12500
7500
2500
C
VEGF-A
HIF-1α
Angiostatin
PROX1
β-tubulin

Figure 1. PROX1 regulates the angiogenesis of human colorectal cancer cells. A. The invasion of HUVECs was significantly decreased in CM of PROX1 siRNA-transfected DLD1 and SW480 cells, compared to the invasion observed in the CM from cells treated with scramble siRNA (P = 0.023 and 0.031, respectively). In contrast, the invasion of HUVECs was significantly increased in pcDNA6-myc-PROX1 transfected DLD1 and SW480 cells, compared to empty-pcDNA6-myc transfected cells (P = 0.031 and P = 0.046, respectively). B. CM from PROX1 siRNA-transfected DLD1 and SW480 cells inhibited the tube formation of HUVECs stronger than CM from cells treated with scramble siRNA (P = 0.008 and 0.048, respectively). In contrast, the tube formation of HUVECs was increased in pcDNA6-myc-PROX1 transfected cells, compared to empty-pcDNA6-myc transfected cells (P < 0.001 and = 0.072, respectively). C. The expression VEGF-A and HIF-1α was down-regulated in all tested cells by PROX1 knockdown. VEGF-A in all tested cells and HIF-1α in DLD1 cells were up-regulated by PROX1 overexpression. The expression of angiostatin was up-regulated by PROX1 knockdown, and down-regulated by PROX1 overexpression in all tested cells. HUVEC; human umbilical vein endothelial cell, SS; scramble siRNA, PS; PROX1 siRNA, WT; wild type, EV; Empty-pcDNA6-myc, PV; pcDNA6-myc-PROX1, CM; conditioned medium, VEGF; vascular endothelial growth factor, HIF-1α; Hypoxia inducible factor-1α; *P < 0.05 *versus* control.

under an inverted phase contrast microscope. Total tube length was analyzed by the WIMtube image analysis platform (WIMASIS GmbH, Munich, Germany).

Western blot analysis

Cells were washed with PBS and then lysed in the RIPA extraction solution (Thermo, Rockford, IL, USA). Total cell extracts were separated on SDS-polyacrylamide gels and transferred to a PVDF membrane (Bio-Rad, Hercules, CA, USA). Protein bands were developed using an enhanced chemiluminescence (ECL) detection system (Amersham, Arlington Heights, IL, USA) and the luminescent image analyzer LAS-4000 (Fujifilm, Tokyo, Japan). Antibodies against the following proteins were used: human PROX1, CD44 (Origene, Rockville, MD, USA); vascular endothelial growth factor (VEGF)-A, matrix metalloproteinase (MMP)-2 and MMP-9, VEGF-C, VEGF-D, β-tubulin (Santa Cruz Biotechnology, CA, USA); hypoxia-inducible factor-1α (HIF-1α), angiostatin (Abcam, Cambridge, UK); zona occludens (ZO)-1, E-cadherin, Vimentin, p-GSK3β, p-Akt, Akt, extracellular signal-regulated kinase (ERK)1/2, p-ERK1/2, p38, p-p38, c-Jun NH2-terminal kinase (JNK), p-JNK (Cell Signaling, Danvers, MA, USA); CD133 (eBioscience, San Diego, CA, USA).

Transwell migration and invasion assays

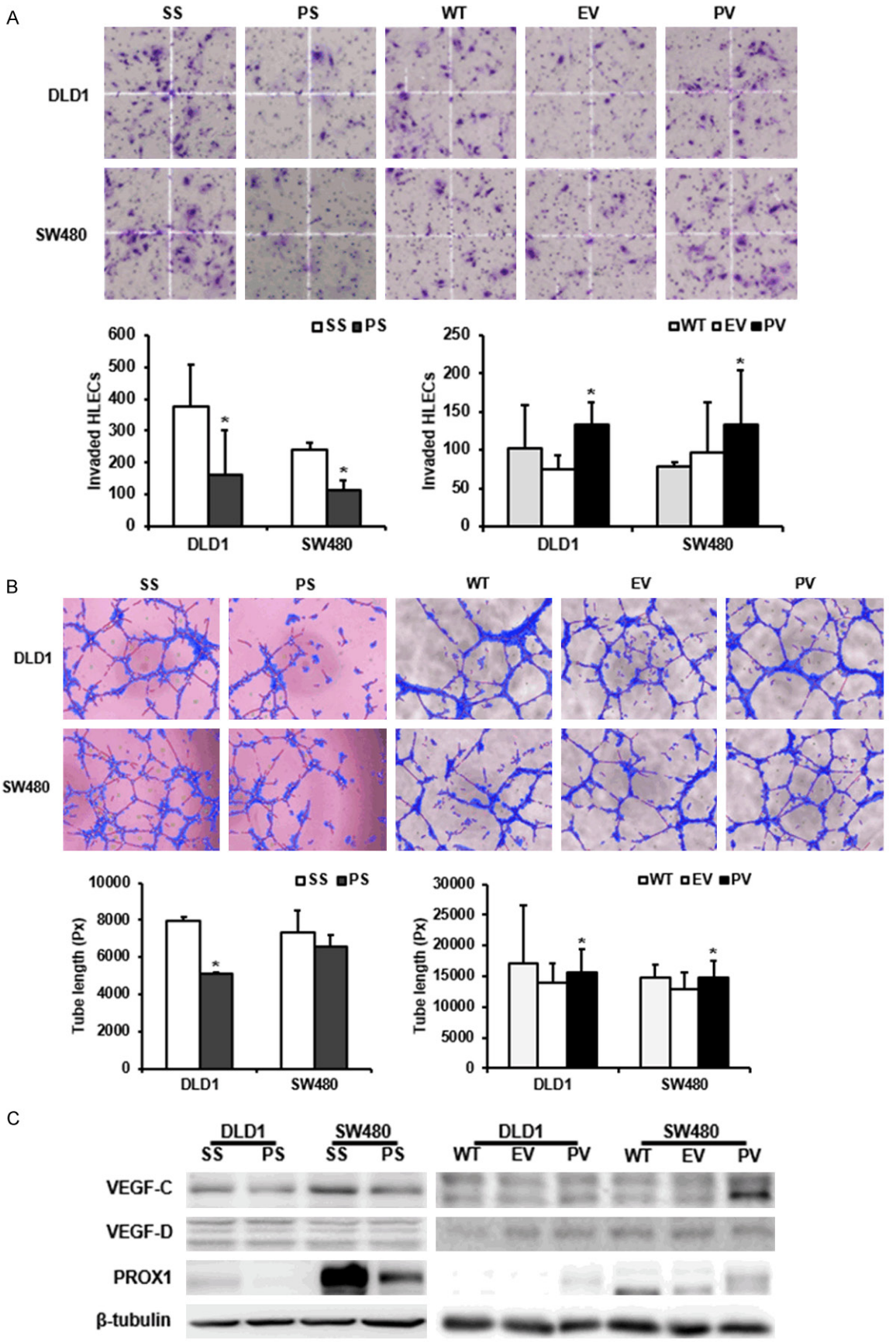
Invasion and migration assays were performed using transwell filter chambers (8.0 μm pores) with or without 1% gelatin coating, respectively. Transfected DLD1 and SW480 cells were placed into the upper chamber. Fibronectin (10 μg/mL) was added as a chemoattractant to 0.2% BSA medium in the lower chamber. After 24 h incubation, cells that migrated and invaded the bottom surface of the upper chamber were fixed with 70% ethanol and stained with the Diff-Quik solution (Sysmex) following the manufacturer's protocol. The stained cells on the bottom surface were counted in 5 selected fields under a light microscope.

Patients and tissue samples

For the histological analysis, we used histopathological specimens obtained from 528 patients operated for colorectal cancer at the Chonnam National University Hwasun Hospital (Jeonnam, Korea) between July 2004 and June 2006. All specimens were embedded in paraffin for the study. None of the patients had received preoperative radiotherapy or chemotherapy. Tissue blocks were selected by viewing original pathologic slides and choosing blocks that showed the junction between the normal colon epithelium and tumor region. Tumor staging was done in accordance with the American Joint Committee on Cancer (AJCC) staging system [29]. Overall survival was calculated from the date of the initial surgery until the follow-up on December 31, 2012. This study was approved by the Institutional review board of the Chonnam National University Hwasun Hospital. All participants gave written consent for their information to be stored in the hospital database and used for research.

Immunohistochemistry

Paraffin tissue sections were dewaxed in xylene and gradually rehydrated. Activity of the endogenous peroxidase was blocked by peroxydase-blocking solution (Dako, Carpinteria, CA, USA) and retrieved with citrate buffer (pH 6.0, Dako). Immunohistochemical reactions were performed using antibodies against the following proteins: human PROX1 (Santa Cruz Biotechnology), Ki-67 (Dakopatts, Glostrup, Denmark), and CD34 (Abcam). We also used the D2-40 antibody (Dakopatts) in primary diluent solution (Invitrogen). Antibody binding was visualized by


A
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Tube length (Px)
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25000
20000
15000
5000
C
VEGF-C
VEGF-D
PROX1
β-tubulin

Figure 2. PROX1 regulates the lymphangiogenesis of human colorectal cancer cells. A. The invasion of HLECs was significantly decreased in CM of PROX1 siRNA-transfected DLD1 and SW480 cells, compared to the effect of CM from cells treated with scramble siRNA (P = 0.044 and 0.025, respectively). In contrast, the invasion of HLECs was significantly increased in pcDNA6-myc-PROX1 transfected DLD1 and SW480 cells, compared to empty-pcDNA6-myc transfected cells (P = 0.006 and 0.019, respectively). B. CM from PROX1 siRNA-transfected DLD1 cells, but not SW480 cells, inhibited the tube formation of HLECs stronger than CM from scramble siRNA-transfected cells (*P* = 0.039 and 0.161, respectively). In contrast, the tube formation of HLECs was significantly increased in pcDNA6-myc-PROX1 transfected DLD1 and SW480 cells, compared to empty-pcDNA6-myc transfected cells (*P* = 0.041 and 0.001, respectively). C. The expression of VEGF-C was down-regulated by PROX1 knockdown, and up-regulated by PROX1 overexpression in all tested cells, but not of VEGF-D. HLEC; human lymphatic endothelial cell, SS; scramble siRNA, PS; PROX1 siRNA, WT; wild type, EV; Empty-pcDNA6-myc, PV; pcDNA6-myc-PROX1, CM; conditioned medium, VEGF; vascular endothelial growth factor, **P* < 0.05 *versus* control.

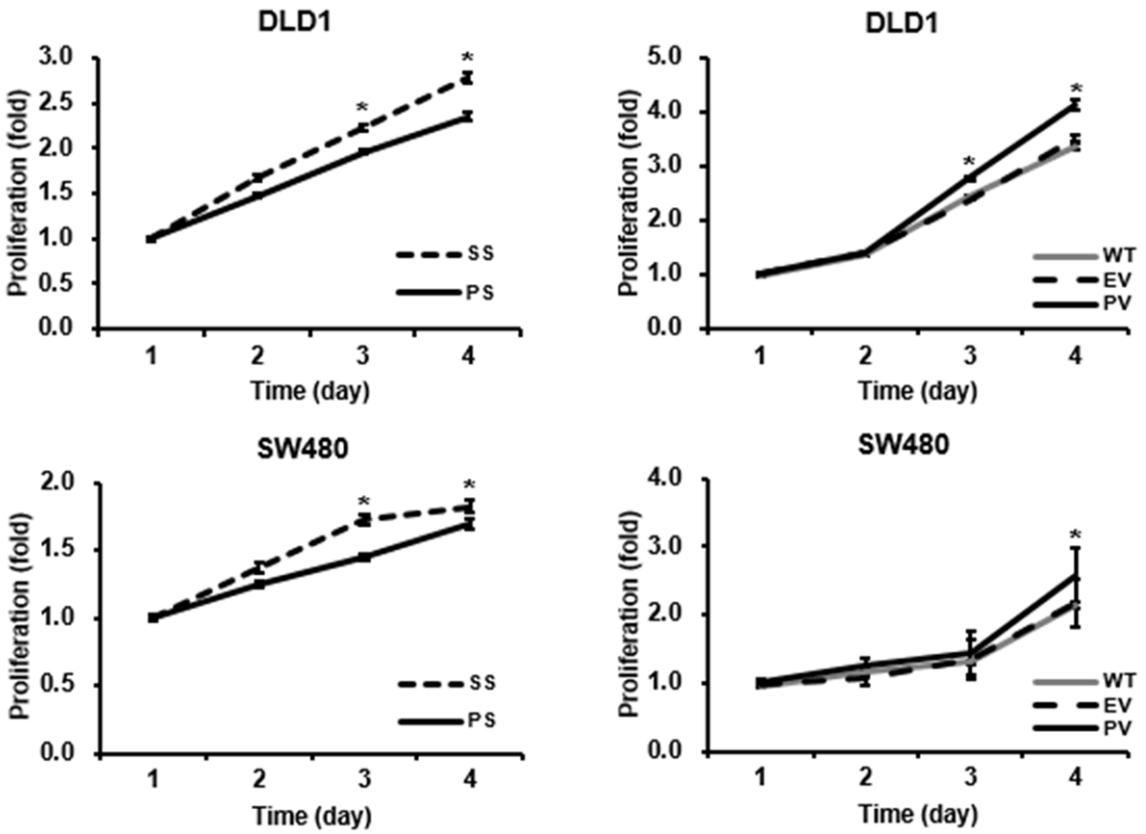

Figure 3. PROX1 enhances the proliferation of human colorectal cancer cells. The number of proliferating cells at days 3 and 4, as determined by changes in absorbance, was significantly decreased in PROX1 siRNA-transfected cultures than in scramble siRNA treated cultures of DLD1 (*P* = 0.019 and 0.005, respectively) and SW480 cells (*P* = 0.018 and 0.022, respectively). In contrast, the number of proliferating cells was significantly increased in pcDNA6-myc-PROX1 transfected DLD1 (*P* = 0.038 and 0.039, respectively) and SW480 cells (*P* = 0.143 and 0.044, respectively), compared to empty-pcDNA6-myc transfected cells. SS; scramble siRNA, PS; PROX1 siRNA, WT; wild type, EV; Empty-pcDNA6-myc, PV; pcDNA6-myc-PROX1. **P* < 0.05 *versus* control.

using Dako Real™ Envision HRP/DAB detection system (Dako). The slides were counterstained with hematoxylin, dehydrated, and mounted.

Evaluation of PROX1 expression

Assessment of PROX1 expression was determined independently by two pathologists who were blind to the knowledge of clinical outcome data. The score discrepancies were discussed to obtain consensus. The staining intensity was graded as follows: 0 (no staining), 1 (weak staining), 2 (moderate staining), 3 (strong staining). The percentage of the stained area was classified as 0 for the absence of positive staining of tumor cells, 1 for positive staining in <

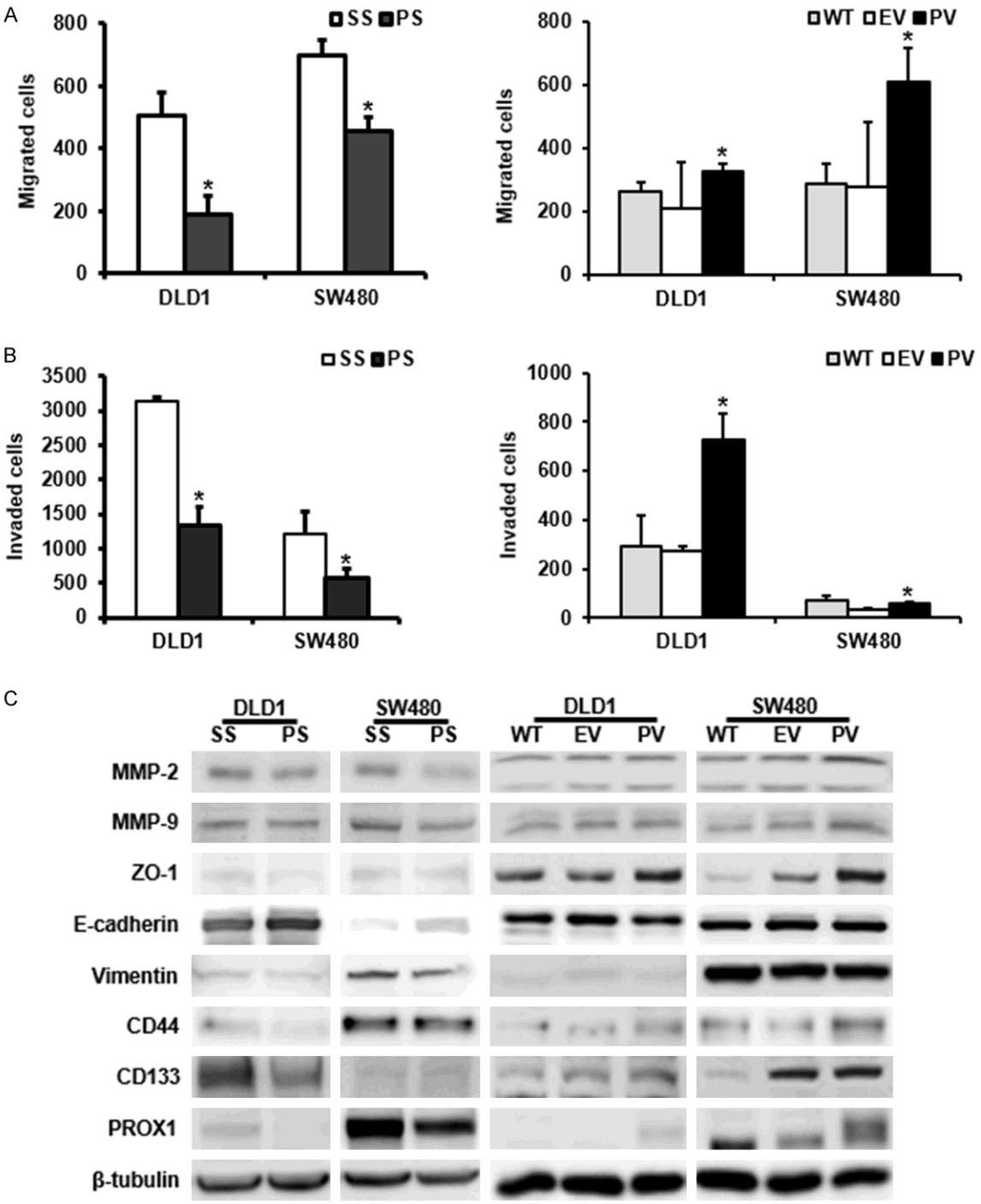

Figure 4. PROX1 promotes EMT in human colorectal cancer cells. A. The number of migratory cells was significantly lower in PROX1 siRNA-transfected than in scramble siRNA-treated DLD1 and SW480 cells (P = 0.001 and 0.017, respectively). In contrast, the number of migratory cells was significantly higher in pcDNA6-myc-PROX1 transfected DLD1 and SW480 cells (*P* = 0.023 and < 0.001, respectively), compared to empty-pcDNA6-myc transfected cells. B. The number of invaded DLD1 and SW480 cells was significantly lower in PROX1 siRNA-transfected cultures than in scramble siRNA-transfected cultures (*P* = 0.006 and 0.038, respectively). In contrast, the number of invaded cells was significantly higher in pcDNA6-myc-PROX1 transfected DLD1 and SW480 cells (*P* = 0.025 and < 0.001, respectively), compared to empty-pcDNA6-myc transfected cells. C. The expression levels of Vimentin, MMP-2, MMP-9, and ZO-1 were down-regulated and the expression level of E-cadherin was up-regulated by PROX1 knockdown. In contrast, the expression levels of MMP-2 and MMP-9 in SW480 cells, and ZO-1 in all tested cells were up-regulated by PROX1 overexpression. However, the expression levels of E-cadherin and Vimentin were not altered in response to

PROX1 overexpression. Expression levels of CD44 and CD133 were decreased by PROX1 knockdown, and increased by PROX1 overexpression. Each bar represents the mean ± SE of 3 experiments. *$P < 0.05$ *versus* control. MMP; matrix metalloproteinase, ZO; zona occludens, SS; scramble siRNA, PS; PROX1 siRNA, WT; wild type, EV; Empty-pcDNA6-myc, PV; pcDNA6-myc-PROX1.

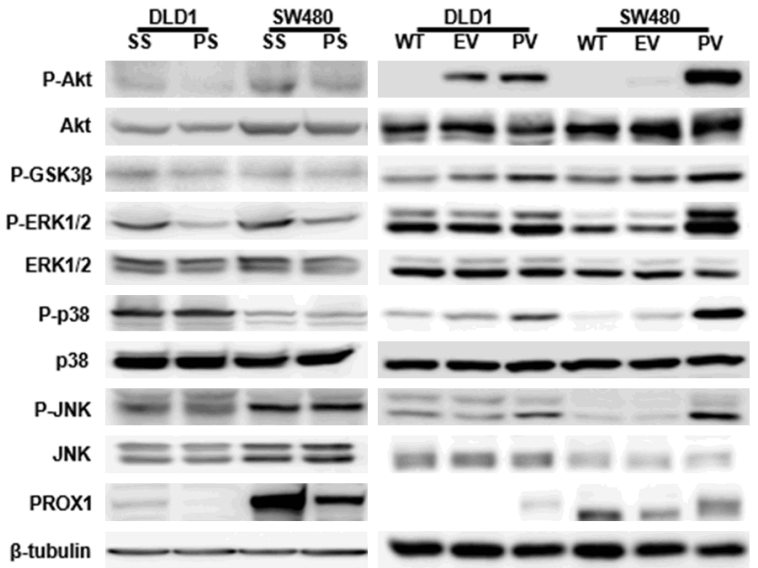

Figure 5. Effect of PROX1 on oncogenic signaling pathways in human colorectal cancer cells. Phosphorylation levels of Akt, GSK3β, and ERK1/2 were decreased in DLD1 and SW480 cells by PROX1 knockdown, while p38 and JNK phosphorylation levels were not altered. In contrast, the phosphorylation levels of Akt, GSK3β, ERK1/2, p38 and JNK were increased by PROX1 overexpression. SS; scramble siRNA, PS; PROX1 siRNA, WT; wild type, EV; Empty-pcDNA6-myc, PV; pcDNA6-myc-PROX1.

10% of the tumor cells, 2 for positive staining in 10% to 50% of the tumor cells, 3 for positive staining in > 50% of the tumor cells. Multiplication of the intensity and percentage scores was used as the final score index. Samples with a total score of ≥ 6 were designated as positive for PROX1 expression, while those with a total score of < 6 were designated as negative for PROX1 expression.

Assessment of tumor cell proliferation

Tumor cell proliferation was determined by immunostaining using an antibody against human Ki-67 protein. A distinct nuclear immunoreactivity for Ki-67 was considered as positive labeling. The Ki-67 labeling index (KI) was defined as the number of Ki-67 positive nuclei per 1000 tumor cell nuclei. Counting was performed on five randomly chosen microscopic fields (40× original magnification) per sample by two independent blinded observers.

Assessment of microvessel (MVD) and lymphovessel density (LVD)

Quantitative analysis of microvessel (MVD) and lymphovessel density (LVD) was performed in sections after immunostaining for CD34 and D2-40, respectively. Immunostained sections were screened at a low-power 40× magnification to identify 3 areas with the highest density of vessels (hot spots) in peritumoral and intratumoral regions. Then, selected hot spots for each case were counted at a higher magnification (400×). The average number of vessels in these 3 areas was defined as section MVD and LVD.

Statistical analysis

Clinicopathological factors and survival curves of colorectal cancer patients were analyzed using Statistical Package for the Social Sciences (Version 15.0; SPSS, Chicago). Correlations between clinicopathological factors and PROX1 expression were assessed using chi-square tests and Fisher's exact test. Patient survival analysis was performed using the Kaplan-Meier method and a log-rank test. Multivariate analysis of prognostic factors was performed using the Cox proportional hazards model. Experimental differences between the PROX1 knockdown or overexpression group and control group were tested with the Student's *t*-test. Differences were considered to be statistically significant if $P < 0.05$.

Results

PROX1 regulates the angiogenesis of human colorectal cancer cells

In all experiments, we used siRNA or pcDNA6-myc vector to control the expression of the

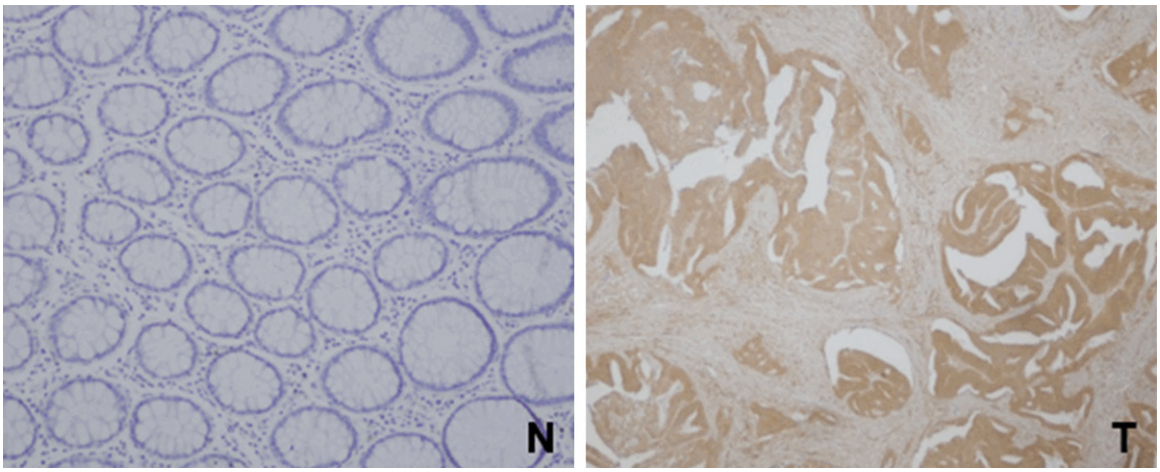

Figure 6. Expression of PROX1 is stronger in human colorectal cancer tissues than in normal colorectal mucosal tissues. In colorectal cancer tissues, immunostaining of PROX1 protein was predominantly observed in the cytoplasm of cancer cells and was not detectable in the tumor stroma. The PROX1 protein showed weak or no immunostaining in normal colorectal mucosa (×200). T; colorectal cancer tissue, N; normal colorectal mucosa.

PROX1 gene. To evaluate PROX1 effects on angiogenesis of HUVECs, we performed Matrigel invasion and tube formation assays using CM from DLD1 and SW480 cells transfected with either siRNA or pcDNA6-myc vector. The invasion of HUVECs was significantly decreased in CM of PROX1 siRNA-transfected DLD1 and SW480 cells, compared to the invasion observed in the CM from cells treated with scramble siRNA (P = 0.023 and 0.031, respectively). In contrast, the invasion of HUVECs was significantly increased in pcDNA6-myc-PROX1 transfected DLD1 and SW480 cells, compared to empty-pcDNA6-myc transfected cells (P = 0.031 and 0.046, respectively) (**Figure 1A**). CM from PROX1 siRNA-transfected DLD1 and SW480 cells exerted a stronger inhibitory effect on the formation of endothelial tubes, than CM from cells transfected with scramble siRNA (P = 0.008 and 0.048, respectively). In contrast, the tube formation of HUVECs was increased in pcDNA6-myc-PROX1 transfected cells, compared to empty-pcDNA6-myc transfected cells (P < 0.001 and = 0.072, respectively) (**Figure 1B**). The angiogenic inducers VEGF-A and HIF-1α were down-regulated in all tested cells by PROX1 knockdown. VEGF-A in all tested cells and HIF-1α in DLD1 cells were up-regulated by PROX1 overexpression. Moreover, the expression of the angiogenic inhibitor angiostatin was up-regulated by PROX1 knockdown, and down-regulated by PROX1 overexpression in all tested cells (**Figure 1C**).

PROX1 regulates the lymphangiogenesis of human colorectal cancer cells

To assess the effects of PROX1 on lymphangiogenesis of HLECs, we performed a similar set of Matrigel invasion and tube formation assays, as described above, utilizing CM from siRNA or pcDNA6-myc vector-transfected DLD1 and SW480 cells. As in the case with HUVECs, the invasion and tube formation of HLECs were significantly decreased in CM from PROX1 siRNA-transfected DLD1 cells (P = 0.044 and 0.039, respectively). However, in SW480 cells, only the invasion of HLECs was negatively affected by CM from PROX1 siRNA-treated cells (P = 0.025 and 0.161 for data on invasion and tube formation, respectively). In contrast, the invasion and tube formation of HLECs was significantly increased in pcDNA6-myc-PROX1 transfected DLD1 (P = 0.006 and 0.041, respectively) and SW480 cells (P = 0.019 and 0.001, respectively), compared to empty-pcDNA6-myc transfected cells (**Figure 2A**, **2B**). The expression of the lymphangiogenic inducer VEGF-C was down-regulated by PROX1 knockdown, and up-regulated by PROX1 overexpression in all tested cells, but not of VEGF-D (**Figure 2C**).

PROX1 enhances the proliferation of human colorectal cancer cells

To determine possible effects of PROX1 on cell proliferation, cell proliferation assays were performed 1, 2, 3, and 4 days after the transfection of cells with siRNA or pcDNA6-myc vector.

Table 1. Correlation between PROX1 expression and clinicopathological parameters of human colorectal cancer

Parameters	Total (n = 528)	PROX1		P-value
		Negative (n = 303)	Positive (n = 225)	
Age (years)				0.348
< 69.5	233	139	94	
≥ 69.5	295	164	131	
Sex				0.711
Male	319	181	138	
Female	209	122	87	
Tumor size (cm)				0.020
< 4.8	275	171	104	
≥ 4.8	253	132	121	
Histologic type				0.011
Differentiated	458	253	205	
Undifferentiated	70	50	20	
Lymphovascular invasion				0.001
Negative	383	237	146	
Positive	145	66	79	
Perineural invasion				0.196
Negative	361	214	147	
Positive	167	89	78	
Stage				0.001
I/II	276	177	99	
III/IV	252	126	126	
Depth of invasion (T)				0.001
T1/T2	111	79	32	
T3/T4	417	224	193	
Lymph node metastasis (N)				0.022
N0	284	176	108	
N1-3	244	127	117	
Distant metastasis (M)				0.187
M0	460	269	191	
M1	68	34	34	

PROX1, Prospero homeobox 1.

As determined by absorbance changes, the number of proliferating cells on days 3 and 4 was significantly decreased in PROX1 siRNA-transfected cultures of DLD1 (P = 0.019 and 0.005, respectively) and SW480 cells (P = 0.018 and 0.022, respectively) than in cultures treated with scramble siRNA. In contrast, the number of proliferating cells was significantly increased in pcDNA6-myc-PROX1 transfected DLD1 (P = 0.038 and 0.039, respectively) and SW480 cells (P = 0.143 and 0.044, respectively), compared to empty-pcDNA6-myc transfected cells (**Figure 3**).

PROX1 promotes EMT in human colorectal cancer cells

To investigate the relationship between PROX1 and EMT in human colorectal cancer cells, the migration and invasion assays were performed. The number of migratory cells was significantly lower among PROX1 siRNA-transfected DLD1 and SW480 cells than in cells treated with scramble siRNA (P = 0.001 and 0.017, respectively) (**Figure 4A**). The number of invaded cells was significantly lower in DLD1 and SW480 cells treated with PROX1 siRNA than in cells transfected with scramble siRNA (P = 0.006 and 0.038, respectively). In contrast, the number of migratory and invaded cells was significantly higher in in pcDNA6-myc-PROX1 transfected DLD1 (P = 0.023 and 0.025, respectively) and SW480 cells (P < 0.001 and < 0.001, respectively), compared to empty-pcDNA6-myc transfected cells (**Figure 4A**, **4B**). To investi gate phenotypic changes induced by EMT, expression levels of EMT-associated genes (MMP-2, MMP-9, ZO-1, E-cadherin, and Vimentin) were also assessed. The expression levels of Vimentin, MMP-2, MMP-9, and ZO-1 were down-regulated and the expression level of E-cadherin was up-regulated by PROX1 knockdown. In contrast, the expression levels of MMP-2 and MMP-9 in SW480 cells, and ZO-1 in all tested cells were up-regulated by PROX1 overexpression. However, the expression levels of E-cadherin and Vimentin were not altered in response to PROX1 overexpression. Next, we investigated a possible role of PROX1 on the expression of cancer stemness markers CD44 and CD133. We found that their expression levels were down-regulated by PROX1 knockdown. Also, the expression levels of CD44 and CD133 were up-regulated by PROX1 overexpression (**Figure 4C**). Our results indicate that PROX1 expression is associated with the induction of molecular and cellular alterations consistent with EMT.

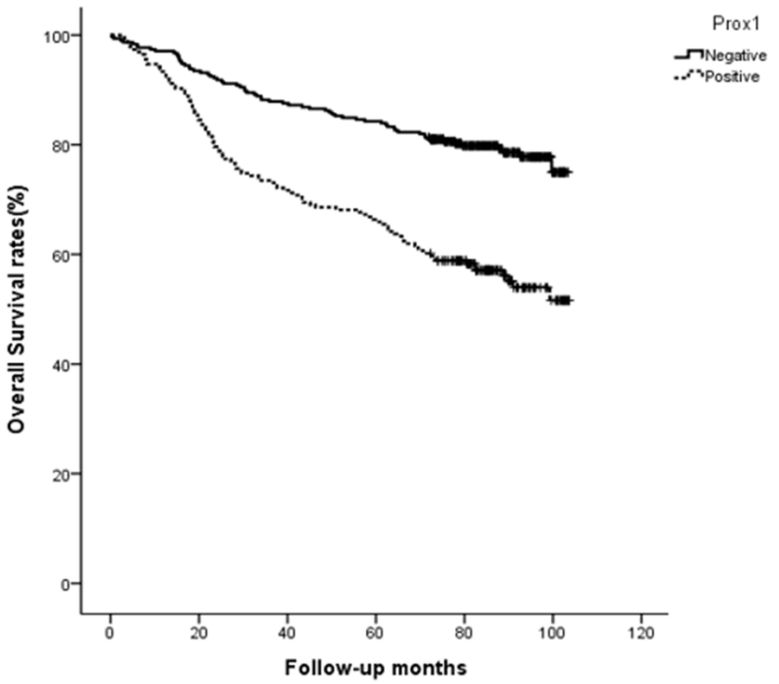

Figure 7. Kaplan-Meier survival curve correlating overall survival with positive (dotted line) and negative (solid line) expression of PROX1. The overall survival of patients with positive PROX1 immunostaining was significantly lower than survival of patients without PROX1-positive tumors (P < 0.001).

Effect of PROX1 on oncogenic signaling pathways in human colorectal cancer cells

To examine whether PROX1 activates intracellular signaling pathways in human colorectal cancer cells, we determined phosphorylation levels of Akt, GSK3β and MAPK signaling proteins using western blotting. We found that phosphorylation levels of Akt, GSK3β, and ERK1/2 were decreased, whereas phosphorylation of p38 and JNK was not altered by PROX1 knockdown in DLD1 and SW480 cells. In contrast, the phosphorylation levels of Akt, GSK3β, ERK1/2, p38 and JNK were increased by PROX1 overexpression in all tested cells (**Figure 5**).

PROX1 protein expression in relation to clinicopathological parameters of human colorectal cancer

To test whether PROX1 protein is associated with human colorectal cancer progression, we evaluated the expression of the PROX1 protein in 528 colorectal cancer tissues by immunohistochemistry. PROX1 expression was higher in colorectal cancer tissues than in normal colorectal mucosa tissues (**Figure 6**). Next, we analyzed survival rates and examined a relationship between PROX1 immunostaining and clinicopathological parameters. PROX1 immunostaining positively correlated with tumor size, differentiation, lymphovascular invasion, stage, depth of invasion, and lymph node metastasis (P = 0.020, = 0.011, = 0.001, = 0.001, = 0.001, and = 0.002, respectively) (**Table 1**). Moreover, the overall survival rate of patients with positive PROX1 immunostaining was significantly lower than that of patients without it (P < 0.001) (**Figure 7**). The association between clinicopathological parameters and prognosis of colorectal cancer is shown in **Table 2**. Patients with positive PROX1 expression had an elevated risk of death after adjustments for age, sex, and tumor size with a hazard ratio (95% C.I.) of 2.283 (1.6503.157).

Correlation between PROX1 protein expression and tumor cell angiogenesis, lymphangiogenesis, and proliferation in human colorectal cancers

All tumor samples underwent immunostaining for CD34, D2-40, and Ki-67 as markers of tumor cell angiogenesis, lymphangiogenesis, and proliferation, correspondingly (**Figure 8**). MVD values for the 528 tumors studied ranged from 23.0 to 429.0 with the mean MVD of 112.3 ± 71.0. The mean MVD value of PROX1 positive tumors was 137.1 ± 83.4, which was significantly higher than the mean MVD of PROX1 negative tumors (P = 0.010). Values of KI for the 528 tumors ranged from 21.9 to 81.8 with the mean KI of 51.5 ± 13.3. The mean KI value of PROX1 positive tumors was 57.2 ± 11.1 and, as in the case of MVD, it was significantly higher than KI of PROX1 negative tumors (P = 0.007). LVD for the set of tumor samples studied ranged from 4.0 to 31.3 with the mean LVD value of 13.4 ± 5.7. There was no statistically significant correlation between PROX1 expression and LVD (P = 0.077) (**Table 3**).

Table 2. Cox multivariate regression of the association between PROX1 immunoreactivity and survival in colorectal cancer adjusted for clinicopathological parameters

Covariate	HR	95% CI	*P*-value
PROX1 expression			
Low	1.000	Ref.	
High	2.283	1.650-3.157	< 0.001
Age			
< 69.5	1.000	Ref.	
≥ 69.5	1.352	0.983-1.860	0.063
Tumor size			
< 4.8	1.000	Ref.	
≥ 4.8	1.508	1.096-2.076	0.012
Histologic type			
Differentiated	1.000	Ref.	
Undifferentiated	2.101	1.415-3.120	< 0.001
Lymphovascular invasion			
Negative	1.000	Ref.	
Positive	1.538	1.113-2.126	0.009
Depth of invasion (T)			
T1/T2	1.000	Ref.	
T3/T4	1.765	0.989-3.152	0.055
Lymph node metastasis (N)			
N0	1.000	Ref.	
N1-3	1.884	1.348-2.635	< 0.001

PROX1, Prospero homeobox 1; HR, Hazard ratio; CI, Confidence interval; Ref, Reference in Cox proportional hazard model.

Discussion

The homeobox gene *PROX1* is important for embryonic development of a number of organs such as the liver, lens, pancreas, central nervous system, and lymphatic system [13]. Recently, it has been shown that PROX1 may exhibit both tumor suppressive and oncogenic activity, depending on the type of cancer. These findings reflect the complexity of PROX1 role in carcinogenesis [13]. PROX1 overexpression inhibits tumor cell proliferation and is associated with well-differentiated tumors and favorable prognosis in hepatocellular carcinoma and pancreatic cancer [14, 16]. In contrast, PROX1 overexpression promotes tumor progression in glioma, vascular endothelial tumors, and colorectal cancer [21-24]. In the latter case, PROX1 overexpression stimulates dysplasia, tumor growth, and malignant progression. PROX1 overexpression is also associated with poor patient outcomes [21, 25-28]. However, the exact mechanisms through which PROX1 regulates tumor progression are still unknown.

Tumor metastases from primary tumors to distant organs develop via the blood and lymphatic vasculature. Experimental and clinical data indicate that dysregulation of angiogenesis and lymphangiogenesis play a crucial role in the development and growth of cancer metastases [7-9]. The relationships between the expression of PROX1 and such phenomena as angiogenesis or lymphangiogenesis in colorectal cancer have not been previously elucidated. In our study, the invasion and tube formation of HUVECs were significantly decreased by PROX1 knockdown, and increased by PROX1 overexpression. Moreover, PROX1 knockdown decreased the expression of the angiogenic factors VEGF-A and HIF-1α, while it increased the expression of the angiostatic factor angiostatin in human colorectal cancer cells. In contrast, PROX1 overexpression showed the increased expression of angiogenic factors and decreased expression of angiostatic factor. These results show that PROX1 may stimulate tumor angiogenesis by controlling the balance between angiogenic and angiostatic factors in human colorectal cancer.

Moreover, we found that the invasion and tube formation of HLECs as well as the expression of VEGF-C were significantly decreased by PROX1 knockdown. In contrast, PROX1 overexpression enhanced the invasion and tube formation of HLECs with increased expression of VEGF-C. This observation suggests that PROX1 is capable of inducing lympahgniogenesis of human colorectal cancer cells. Previous studies reported an association between the expression of lymphangiogenic inducers, such as VEGF-C, VEGF-D, and VEGFR-3, and cancer progression including lymph node metastasis [8, 9].

The dysregulation of cell survival, migration, and invasion is a principal hallmark of cancer cells [4-6]. EMT is a fundamental process in embryogenesis, in which cells lose epithelial features and acquire mesenchymal properties. However, it is also a common early step in the process of metastasis in a variety of cancers. Accumulating evidence suggests that cancer-associated EMT strongly correlates with the stage of

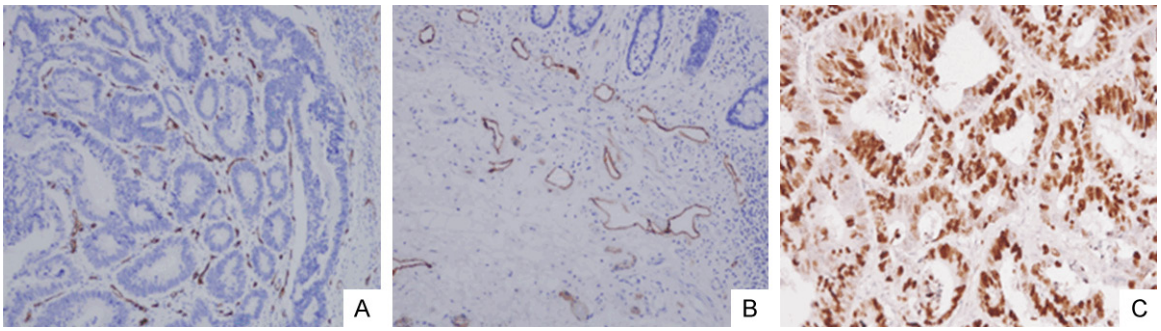

Figure 8. Representative photomicrograph showing positive immunohistochemical staining of CD34, D2-40, and Ki-67 in human colorectal cancer. A. Immunostaining of CD34. B. Immunostaining of D2-40. C. Immunostaining of Ki-67.

Table 3. Correlation between PROX1 expression and angiogenesis, lymphangiogenesis, and tumor cell proliferation in human colorectal cancer

Parameters (Mean ± SD)	Total (*n* = 528)	PROX1 expression		*P*-value
		Negative (*n* = 303)	Positive (*n* = 225)	
MVD	112.3 ± 71.0	91.0 ± 50.1	137.1 ± 83.4	0.010
LVD	13.4 ± 5.7	11.9 ± 4.7	15.4 ± 6.4	0.077
KI	51.5 ± 13.3	46.2 ± 13.3	57.2 ± 11.1	0.007

PROX1, Prospero homeobox 1; SD, Standard deviation; KI, Ki-67 labeling index; MVD, Microvessel density; LVD, Lymphatic vessel density.

the tumor development, metastasis, and unfavorable clinical outcome [10-12]. Previously, it has been suggested that PROX1 promotes EMT and tumor progression in human colorectal cancer [25]. In our study, PROX1 knockdown suppressed tumor cell proliferation, migration, and invasion. Also, PROX1 overexpression enhanced tumor cell proliferation, migration and invasion. In addition, we showed that PROX1 knockdown increased the expression level of the epithelial marker E-cadherin and decreased expression levels of mesenchymal markers including Vimentin, MMP-2, MMP-9, and ZO-1, leading to tumor cell migration and invasion. PROX1 overexpression increased the expression levels of MMP-2, MMP-9 and ZO-1, but the expression levels of E-cadherin and Vimentin were not altered in response to PROX1 overexpression.

Cancer stem cells are a small set of tumor-initiating cells that exhibit stem cell properties such as extensive proliferative capacity, pluripotency, high metastatic potential, and resistance to therapy [30-32]. Recently, several reports demonstrated a convincing link between EMT and cancer stem cells as well as the association of these factors with tumor progression and treatment resistance [33-35]. In our study, PROX1 knockdown decreased the expression level of cancer stemness markers CD44 and CD133. In contrast, PROX1 overexpression increased the expression level of CD44 and CD133.

Akt, GSK3β and MAPKs signaling cascades are known to be involved in motility, survival, EMT, angiogenesis, and lymphangiogenesis of various human cancer cells [36-38]. We evaluated the impact of PROX1 expression on oncogenic signaling pathways. In our study, phosphorylation levels of Akt, GSK3β, and ERK1/2 were decreased by PROX1 knockdown. In contrast, the phosphorylation levels of Akt, GSK3β, ERK1/2, p38 and JNK were increased by PROX1 overexpression.

Next, we examined relationships between PROX1 expression and clinicopathological parameters of human colorectal cancer. We found that PROX1 expression was higher in human colorectal cancer tissues than in normal colorectal mucosa tissues. PROX1 expression positively and significantly correlated with tumor size, extent of differentiation, lymphovascular invasion, depth of invasion, lymph node metastasis, cancer stage, and poor survival. Furthermore, patients with PROX1-positive tumors had an elevated risk of death after adjustments for age, sex, and tumor size. PROX1 expression has been previously associated with poor differentiation and unfavorable patient outcome in human colorectal cancer [21]. Therefore, those results and our present data suggest that PROX1 plays an important role in carcinogenesis and progression of

colorectal cancer. Therefore, PROX1 may serve as a potential prognostic marker and a molecular target for treatment in human colorectal cancer.

Finally, we analyzed the relationship between PROX1 expression and angiogenesis, lymphangiogenesis, and tumor cell proliferation in human colorectal cancer tissues to confirm the results of studies that utilized human colorectal cancer cell lines. We found that mean MVD and KI values of PROX-1 positive tumors were significantly higher than those of PROX-1 negative tumors. However, there was no significant correlation between PROX1 expression and LVD. Thus, these observations corroborated results of *in vitro* studies and confirmed that PROX1 can promote cell proliferation and angiogenesis *in vivo*.

In summary, our experiments *in vitro* showed that PROX1 enhanced angiogenesis, lymphangiogenesis, proliferation, migration, invasion, and EMT of human colorectal cancer cells. *In vivo* studies demonstrated that positive immunohistochemical staining of archival surgical resection specimens of colon cancer for PROX1 positively and significantly correlated with tumor size, extent of differentiation, lymphovascular invasion, depth of invasion, lymph node metastasis, cancer stage, MVD, KI, and poor survival. Collectively, these observations indicate that PROX1 affects tumor progression by regulating angiogenesis and tumor cell proliferation in colorectal cancer.

Disclosure of conflict of interest

None.

Authors' contributions

YEJ conceived and designed the experiments. YLP, EM, NK, SYP, CYO performed the experiments. YEJ, YLP, EM, SBC, DSM, WSL carried out data analysis. SBC, DSM, WSL, SSK contributed reagents/materials/analysis tools. YEJ wrote the paper.

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