

Prospero Homeobox 1 Promotes Epithelial–Mesenchymal Transition in Colon Cancer Cells by Inhibiting E-cadherin via miR-9

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Abstract

Purpose: Prospero homeobox 1 (PROX1) has been shown to function as a tumor suppressor in various types of cancer. However, it promotes colon cancer progression. The aim of this study is to clarify the underlying mechanism by which PROX1 regulates tumorigenicity of colon cancer.

Experimental Design: Association of PROX1 and clinicopathological features was studied by immunohistochemical staining. Pri-miR-9-2 and miR-9 were detected by quantitative real-time PCR. Assays of cell invasion, adhesion, and matrix metalloproteinase activity were used to study PROX1-mediated epithelial–mesenchymal transition (EMT).

Results: PROX1 was overexpressed in 43% (59/136) of colon cancer tissues and its expression was correlated with E-cadherin downregulation ($P = 0.00005$), advanced tumor staging ($P = 0.005$), and lymph node metastasis ($P = 0.00009$). Enforced expression of PROX1 in DLD-1 cells caused downregulation of E-cadherin and integrins and attenuated cell adhesion. These cells showed increase of matrix metalloproteinase activity and invasive ability. Conversely, knockdown of PROX1 in SW620 cells restored E-cadherin protein expression and reduced invasiveness. Unexpectedly, repression of E-cadherin by PROX1 was not mediated by transcriptional inhibition. We found that PROX1 bound to miR-9-2 promoter and triggered its expression to suppress E-cadherin 3'UTR reporter activity and protein expression. Anti-miR-9 restored E-cadherin in SW620 cells, whereas precursor miR-9 inhibited E-cadherin in PROX1-knockdown cells. The miR-9 level was higher in tumor tissues with high PROX1/low E-cadherin than that of tumor tissues with low PROX1/high E-cadherin.

Conclusions: Our results provide mechanistic insights by which PROX1 promotes EMT and colon cancer progression. Targeting of PROX1-mediated oncogenic activity may be helpful for the treatment of colon cancer. *Clin Cancer Res*; 18(23); 6416–25. ©2012 AACR.

Introduction

PROX1 is an evolutionally conserved transcription factor that controls the fate and differentiation of neuron precursor cells, lymphatic endothelial cells, retinal progenitor cells, and hepatocytes (1–4). The *Drosophila* counterpart of PROX1, *Prospero*, has been shown to function as a brain

tumor suppressor. Recently, studies in human cancers suggest a similar role. Downregulation or loss-of-function of PROX1 is caused by mRNA mutation, epigenetic silencing and genomic deletions (5–11). However, PROX1 play a tumor-promoting role in colon cancer, malignant astrocytic glioma and kaposiform hemangioendothelioma (12–14). The distinct function of PROX1 in human cancers is largely unclear.

In colon cancer, PROX1 promotes the transition from benign to highly dysplastic phenotype (12). Inhibition of PROX1 reduces growth of human colon cancer xenografts and intestinal adenomas in *Apc*^{min/+} mice. In addition, PROX1 was identified to be a direct and dose-dependent target of the β -catenin/T-cell factor (TCF) signaling pathway that deregulation is found in the majority of human colon cancers. More importantly, upregulation of PROX1 by β -catenin/TCF is cell context-dependent because this signaling pathway does not control PROX1 expression in normal or transformed hepatocytes. Another evidence supporting the oncogenic role of PROX1 in colon cancer came from the study of estrogen receptor β (15). It was shown that

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Translational Relevance

Metastatic disease is the major cause of death in colorectal cancer patients. Epithelial–mesenchymal transition (EMT)-like dedifferentiation of the tumor cells is one of main characters for malignant phenotype. Here, we identify that Prospero homeobox1 (PROX1) plays a critical role in promoting EMT to trigger malignant phenotype formation. PROX1 directly drives the transcriptional expression of miR-9-2 and subsequently suppresses E-cadherin protein level via posttranscriptional regulation. As a functional consequence, we also show that overexpression of PROX1 decreases the expression of integrins and increases invasion ability. More importantly, tumor array data indicate that PROX1 expression is significantly correlated with E-cadherin downregulation, advanced stage, and lymph node metastasis in colon cancer patients. We conclude that PROX1 functions as an oncogene in colon cancer and promotes cell invasiveness by inhibiting E-cadherin via miR-9. These results represent a clinically relevant mechanism in colon cancer progression and an attractive target for therapy.

expression of estrogen receptor β in colon cancer induced anti-inflammatory and antitumorigenic networks and significantly attenuated the expression of the proinflammatory cytokine IL6. In addition, estrogen receptor β also exhibited its protective role against the development of colon cancer by suppressing PROX1 and its downstream target genes (15). These data support a tumor-promoting role of PROX1 in colon cancer. However, PROX1-mediated oncogenic pathway is still unclear.

Epithelia–mesenchymal transition (EMT) is a biologic process that induces a polarized epithelial cell to a mesenchymal phenotype with significant increase of extracellular matrix production, cytoskeleton modulation, migratory capability, and invasiveness (16). EMT has been classified into 3 types that linked with embryogenesis and organ development (type 1), tissue regeneration and organ fibrosis (type 2), and cancer progression and metastasis (type 3). One of the most important characters of EMT is the downregulation of E-cadherin that leads to attenuation of cell–cell junction and gain of migratory behavior. In this study, we address the effect of PROX1 on the control of EMT in colon cancer cell lines and elucidate the underlying mechanism. In addition, the clinical significance of PROX1 expression is studied in human tumor tissues.

Materials and Methods

Cell culture and reagents

All cell lines were obtained from the cell bank of the National Health Research Institute (Miaoli, Taiwan). SW480 and SW620 cells were grown in Liebovitz L-15 medium. HT-29 and HCT-116 cells were cultured in

McCoy's 5A medium containing 2 mmol/L glutamine. WiDr and SK-Hep-1 cells were maintained in Eagle's minimum essential medium and DLD-1 cells were grown in RPMI-1640 medium. All medium contained 10% FBS and antibiotics. Anti-PROX1 antibody was purchased from Upstate Biotechnology; anti-integrins (including $\alpha 4$, $\alpha 5$, αV , $\beta 1$, $\beta 3$, $\beta 4$, and $\beta 5$) antibodies were purchased from Cell Signaling Technology; anti-E-cadherin was obtained from BD Biosciences. Pre-miR-9 precursor and miR-9 inhibitor were purchased from Ambion. TaqMan Pri-miRNA assay kit for the quantification of Pri-miR-9-2 was purchased from Applied Biosystems.

Plasmids and transfection

pcDNA3.0-FLAG-PROX1 was kindly provided by Dr. Xie YH (Shanghai Institute for Biological Sciences). pGL2 basic-E-cadherin promoter –1,008/+49 was generously provided by Dr. Y.S. Chang (Chang G.ang University, Taiwan). All transfections were carried out using Invitrogen Lipofectamine 2000 Transfection Reagent.

Immunoblotting and immunofluorescence analyses

For immunoblotting, cells were sonicated in radioimmunoprecipitation assay buffer containing (50 mmol/L Tris/HCl, pH 7.5, 150 mmol/L NaCl, 1 mmol/L EDTA, 0.5 M sucrose, 0.25% sodium deoxycholate, 10% glycerol, 1% NP-40, and protease inhibitor mixture), and cellular debris were removed by centrifugation. Protein concentration was determined by the BCA protein Assay kit from Pierce. Equal amounts of cellular proteins were subjected to SDS-PAGE separation, transferred to nitrocellulose, and immunoblotting was conducted by using the appropriate antibodies. Immunofluorescence staining was conducted as described previously (17).

Reverse transcription-polymerase chain reaction and Pri-miR and miRNA quantification

For miRNA quantification, the first strand cDNA synthesis and quantitative real-time PCR (qRT-PCR) were conducted by using NCode TM miRNA First-Strand cDNA synthesis and qRT-PCR kits according to the manufacturer's instruction (Invitrogen). The primer sequences used were shown in Supplementary Fig. S1. For the quantification of pri-miR-9-2, we used TaqMan Pri-miRNA assay system.

Chromatin immunoprecipitation assay and reporter activity assay

Cells were harvested and chromatin immunoprecipitation assay was conducted as described previously (18). Specific primers for the region contained the PROX1 binding site in human *miR-9-2* gene promoter are forward: 5'-CTCTTGCCAGACTCCAGGTC-3' and reverse: 5'-TCCACA-ATTTGGCAGTC AAA-3'. E-Cadherin promoter activity assay were conducted as described previously (19).

Cell invasion, cell adhesion assay, and MMP activity

In brief, *in vitro* invasion assay was conducted by using 24-well transwell units with polycarbonate filters (pore size

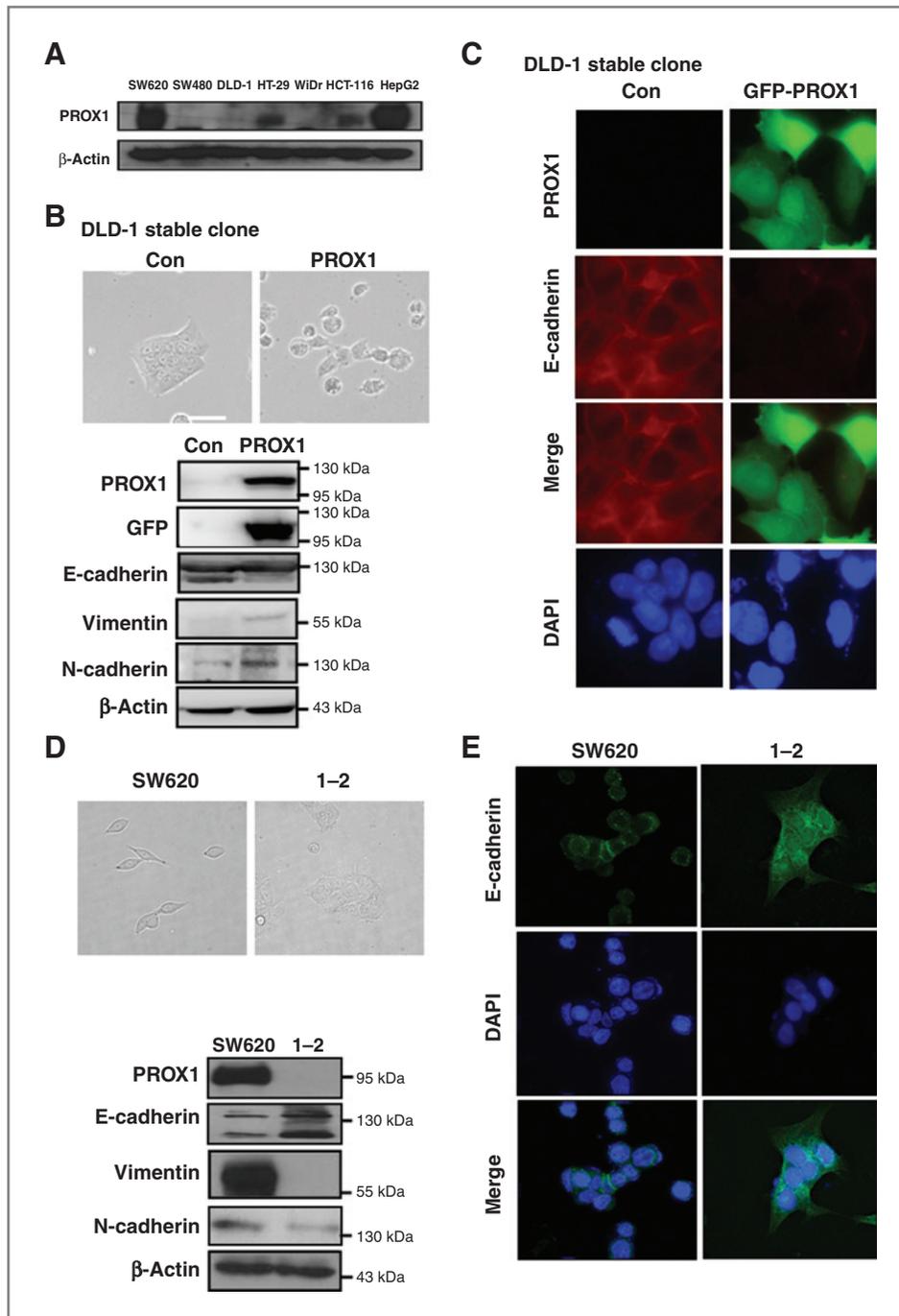


Figure 1. PROX1 inhibits E-cadherin and induces EMT in colon cancer cells. **A**, expression of PROX1 in human colon cancer cell lines. HepG2 human hepatoma cell line was used as a positive control. **B**, induction of mesenchymal morphology and markers in DLD-1 cells after expression of GFP-PROX1. **C**, immunofluorescent staining showed a reduction of E-cadherin protein at cell-cell junction of PROX1-transfected stable clone. 4', 6-diamidino-2-phenylindole (DAPI) staining (blue) was used for the demonstration of cell nuclei. **D**, change of morphology and expression of EMT-related markers in SW620 cell line and a stable cell line (1-2) generated by knockdown of PROX1 in SW620 cells. **E**, immunofluorescent staining showed the increase of E-cadherin protein level at plasma membrane and cell-cell junction of 1-2 cells.

8 μ m) coated on the upper side with 1% gelatin (Sigma). Cell adhesion assay was conducted by coating Laminin, fibronectin, and collagen IV in the 96-well plate. Cells were seeded at 5,000 cells per well and incubated for 30 minutes at 37°C. After incubation, invaded cells and adhered cells were fixed with 10% formaldehyde and stained with 0.05% crystal violet. Matrix metalloproteinase (MMP) activity was studied by using the procedures as described previously (20).

Tumor tissue arrays and immunohistochemical staining

Five tissue arrays contained 136 colon tumor tissues were used for immunohistochemical staining. This study was approved by the Medical Ethics and the Human Clinical Trial Committee of Chang Gung Memorial Hospital-Kaohsiung Medical Center. Tissues were dewaxed and blocked for 5 minutes with 3% hydrogen peroxide to deprive the endogenous peroxidase activity. After antigen

Table 1. Associations of PROX1 and E-cadherin with clinicopathologic variables

Variables	n	PRO X1		P	E-cadherin		P
		Low n (%)	High n (%)		Low n (%)	High n (%)	
Sex							
Male	84	46 (55)	38 (45)	0.598	54 (64)	30 (26)	0.347
Female	52	31 (60)	21 (40)		38 (73)	14 (27)	
Age							
≥60	93	53 (57)	40 (43)	1.000	65 (64)	28 (36)	0.435
<60	43	24 (56)	19 (44)		27 (73)	16 (27)	
Dukes stage							
A	9	9 (100)	0 (0)	0.005	1 (11)	8 (89)	0.0002
B	69	42 (60)	27 (40)		45 (65)	24 (35)	
C	58	26 (44)	32 (56)		46 (79)	12 (21)	
Size							
≥5 cm	70	36 (51)	34 (49)	0.229	44 (63)	26 (37)	0.279
<5 cm	66	41 (62)	25 (38)		48 (75)	18 (25)	
Regional lymph node metastasis							
Number							
0	78	57 (68)	21 (32)	0.000009	43 (55)	35 (45)	0.00002
1	20	10 (50)	10 (50)		13 (65)	7 (35)	
≥2	38	10 (24)	28 (76)		36 (94)	2 (8)	
E-cadherin							
High	77	56 (72)	21 (28)	0.00005			
Low	59	22 (37)	37 (62)				

retrieval with microwave, tissues were subjected for primary antibody incubation. To avoid the staining bias, we carried out doubling staining of PROX1 and E-cadherin on the same tumor section. PROX1 was stained with brown color by using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and diaminobenzidine. E-cadherin was detected by alkaline phosphatase-conjugated goat anti-mouse secondary antibody and Fast Red. The staining intensity of immunohistochemistry (IHC) section was quantified using the TissueFAXS system and was scored as: no staining (0), light positive staining (1), medium positive staining (2), and strong positive staining (3). The positive stained area was scored as: less than 5% (0); 5% to 25% (i); 26% to 50% (ii); 51% to 75% (iii), and more than 75% (iv). A final score was calculated by multiplying the intensity score by expression score and the rating (from 0 to 12) was determined for each case. Tumors were defined as high expression when the final score more than 4. Tumors with a final score 4 or less were defined as low expression.

Statistical analysis

The associations between PROX1 and E-cadherin with clinicopathological parameters were done by using χ^2 test. The associations between different groups of cell-based experiments were studied by Student *t* test. Statistical significance was defined as $P < 0.05$.

Results

PROX1 regulates EMT in colon cancer cells

Because PROX1 was shown to promote colon cancer progression, we first tested the possibility that PROX1 might be involved in the control of EMT in colon cancer cells. Screening of PROX1 expression in various colon cancer cell lines revealed that this transcription factor was detectable in SW620, HT29, and HCT-116 cells, whereas it was very low or undetectable in DLD-1, WiDr, and SW480 cells (Fig. 1A). We established a PROX1-overexpressing stable clone from DLD-1 cells and compared with the control vector clone. Our data showed that PROX1 induced a morphologic change from epithelial type to mesenchymal (or spindle) type and caused down-regulation of E-cadherin and upregulation of N-cadherin and vimentin (Fig. 1B). Similar alterations of EMT proteins were also found in PROX1-transfected SW480 cells (Supplementary Fig. S2). Immunofluorescent staining showed a reduction of E-cadherin protein at cell-cell junction of PROX1-overexpressing cells (Fig. 1C). We also generated a PROX1 knockdown stable clone by short hairpin RNA from and named it as 1-2 cell line. Knockdown of PROX1 induced a morphologic change from mesenchymal type to epithelial type and reduced the protein level of vimentin and N-cadherin (Fig. 1D). In addition, a significant increase of E-cadherin protein was observed in 1-2 cells and E-cadherin was detected

at cell-cell junction. Conversely, little membrane-associated E-cadherin was observed in SW620 cells (Fig. 1E).

PROX1 expression is associated with reduced E-cadherin in colon cancer tissues and is linked with tumor stage and lymph node metastasis

We next studied the association of PROX1 and E-cadherin by tumor array experiments to confirm the results of our cell-based study. To avoid the staining bias, we carried out doubling staining of PROX1 and E-cadherin on the same tumor section. PROX1 was stained with brown color by using horseradish peroxidase-conjugated goat anti-rabbit secondary antibody and diaminobenzidine. E-cadherin was detected by alkaline phosphatase-conjugated goat anti-mouse secondary antibody and Fast Red. Representative figures of tumor tissues with high PROX1/low E-cadherin (Supplementary Fig. S3A) or low PROX1/high E-cadherin (Supplementary Fig. S3B) were shown. Our results showed that PROX1 expression is associated with reduced E-cadherin ($P = 0.00005$, Table 1). In addition, high PROX1

expression is associated with regional lymph node metastasis ($P = 0.000009$) and linked with Dukes' stage ($P = 0.005$). Reduced E-cadherin is also significantly correlated with these 2 clinicopathological parameters (Table 1).

Effect of PROX1 on integrin expression and matrix metalloproteinase activity

The effect of PROX1 on cell invasiveness was addressed. Our data showed that expression of PROX1 in DLD-1 cells reduced cell adhesion to various extracellular matrix proteins including type IV collagen, laminin, and fibronectin (Fig. 2A). In addition, PROX1 reduced cell-adhesion ability and attenuated integrin $\beta 1$ and $\beta 4$ expression. However, no effect on $\beta 3$, $\alpha 5$, and αv expression was found (Fig. 2B). Invasive ability was also enhanced by PROX1 in these cells (Fig. 2C). This increase was accompanied with an upregulation of MMPs in the conditioned medium of PROX1-transfected cells (Fig. 2D). On the contrary, adhesive ability of PROX1-knockdown 1-2 cells was increased (Fig. 2E). As expected, 1-2 cells exhibited reduced MMPs activity and

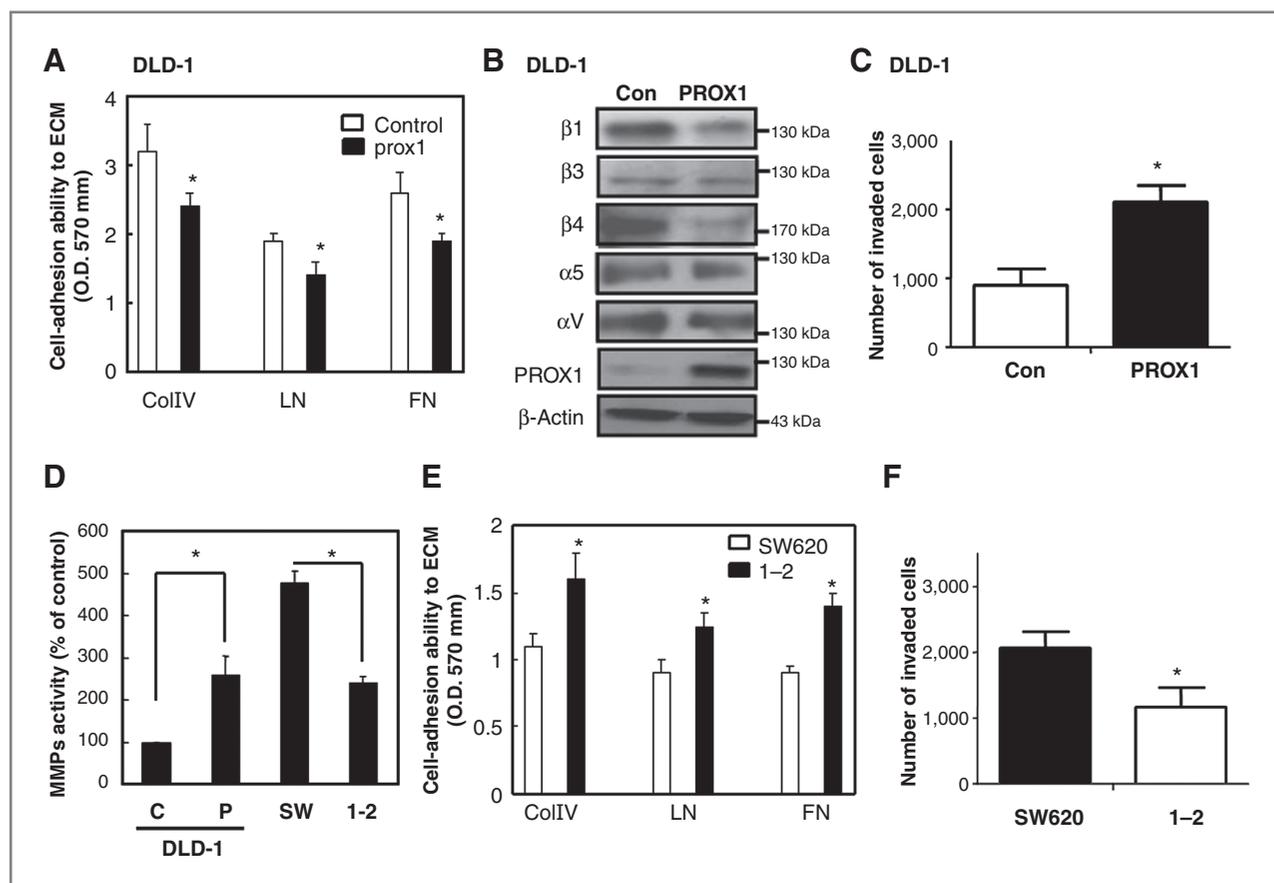
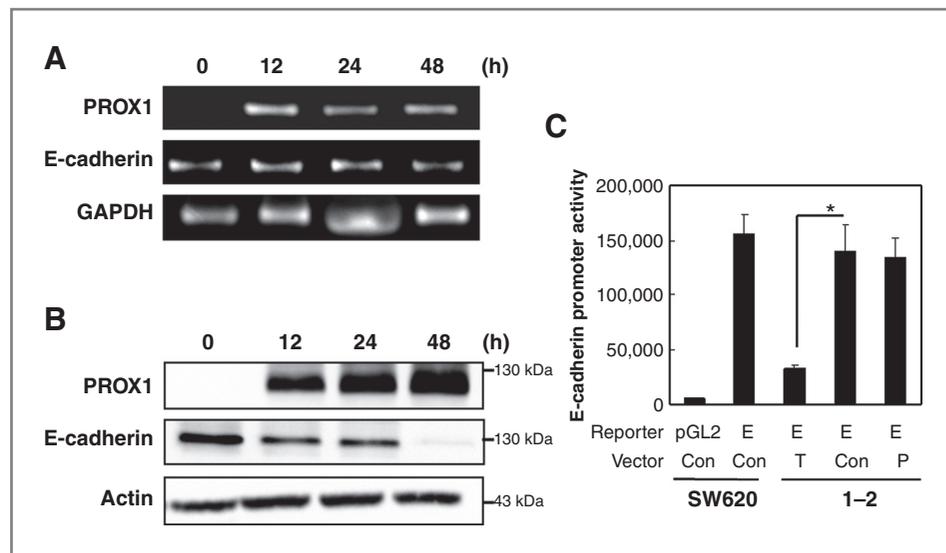


Figure 2. Regulation of cell adhesion, integrin expression, invasion, and MMP activity by PROX1. A, enforced expression of PROX1 in DLD-1 cells reduced their adhesion to collagen IV (ColIV), laminin (LN), and fibronectin (FN). $^*P < 0.05$. B, PROX1 inhibited $\beta 1$ and $\beta 4$ expression in DLD-1 cells. C, PROX1 increased the invasive ability of DLD-1 cells. Transwell assays were conducted and the invaded cell number was counted at 24 hours after seeding of cells. Results of 3 independent assays were expressed as mean \pm SE. $^*P < 0.05$. D, MMP activities in the conditioned medium of DLD-1 cells transfected with control vector (C) or PROX1 expression vector (P) were studied by ELISA assays. The activity of DLD-1 cells transfected with control vector was defined as 100%. Similarly, MMP activities of SW620 cells or 1-2 cells were determined and results from 3 independent assays were expressed as mean \pm SE. $^*P < 0.05$. E, adhesion of SW620 and 1-2 cells to ColIV, LN, and FN. F, invasive ability of SW620 and 1-2 cells was detected as described in C. $^*P < 0.05$.

Figure 3. PROX1 inhibits E-cadherin via a posttranscriptional regulation. **A**, enforced expression of PROX1 did not significantly affect E-cadherin mRNA level in DLD-1 cells. **B**, E-cadherin protein level was downregulated quickly (around 12 hours) after PROX1 induction. **C**, E-cadherin promoter activity was not regulated by PROX1. SW620 and 1–2 cells were cotransfected with empty reporter vector (pGL2) or E-cadherin promoter reporter (E) with control vector (Con, pcDNA3.0), TWIST (T), or PROX1 (P) expression vectors. *, $P < 0.05$.



invasiveness (Fig. 2D and F). These data suggested that PROX1 enhances invasiveness by regulating integrins and MMPs.

PROX1 represses E-cadherin via a posttranscriptional mechanism

E-cadherin is an important controller of EMT, therefore, we studied the mechanism by which PROX1 inhibited its expression. As shown in Fig. 3A and B, a significant induction of PROX1 mRNA and protein was found at 12 h after vector transfection. However, E-cadherin mRNA level was not affected. Conversely, E-cadherin protein level was declined at 12 hours and was almost undetectable at 48 hours. These data suggested that reduction of E-cadherin by PROX1 was not caused by transcriptional repression. To confirm that PROX1 controlled E-cadherin via a posttranscriptional mechanism, we conducted promoter activity assay. As shown in Fig. 3C, E-cadherin promoter activity was similar in SW620 cells and PROX1-repressed 1–2 cells (Fig. 3C). This is not because of the failure of transfection because TWIST, a direct transcriptional repressor of E-cadherin, indeed effectively suppressed promoter activity in 1–2 cells. However, PROX1 did not affect E-cadherin promoter activity under the same experimental condition. These results suggested that PROX1 acts via a posttranscriptional mechanism to repress E-cadherin.

Expression of miR-9 in colon cancer cell lines and its regulation by PROX1

MicroRNAs are known to inhibit gene expression posttranscriptionally by inducing translational blockage or mRNA degradation. A recent study showed that E-cadherin is a direct target of miR-9 (21). We tested whether PROX1 might increase miR-9 expression to repress E-cadherin. Our quantitative RT-PCR result showed that miR-9 was detectable in all colon cancer cell lines studied (Fig. 4A). Interestingly, an averaged 15-fold of increase of miR-9 was found

in SW620 cells when compared with that of DLD-1 cells. In consistent with our hypothesis, ectopic expression of PROX1 in DLD-1 cells upregulated miR-9 by 3- to 4-fold (Fig. 4B). In addition, PROX1 also increased miR-9 in WiDr cells that do not express PROX1 (Supplementary Fig. S4). We next studied whether miR-9 is a direct transcriptional target of PROX1. MiR-9 in cells is encoded by 3 genes *miR-9-1*, *miR-9-2*, and *miR-9-3* that located at different chromosomes. Previous studies showed that *miR-9-1* and *miR-9-3* are frequently hypermethylated in colon cancer (22, 23). Therefore, we focused on the *miR-9-2* gene and our *in silico* prediction analysis revealed potential PROX1 binding sites in *miR-9-2* gene promoter (data not shown). ChIP assays indeed showed that PROX1 bound to *miR-9-2* promoter and this binding was significantly reduced in 1–2 cells (Fig. 4C). In addition, miR-9 level of 1–2 cells was significantly lower than that of SW620 cells (Fig. 4D). To confirm PROX1 increased miR-9 via transcriptional activation, we quantified pri-miR-9-2 level by qRT-PCR and found that PROX1 up-regulated pri-miR-9-2 by 2.5-fold in DLD-1 cells (Fig. 4B). In consistent with our hypothesis, the magnitude of reduction of pri-miR-9-2 was similar that of the reduction of miR-9 in 1-2 cells (Fig. 4D). These data strongly supported that PROX1 increased miR-9 via transcriptional stimulation. However, it should be noted that regulation of miR-9 by PROX1 is cell type-dependent because PROX1 could not up-regulate miR-9 in SK-HEP-1 human hepatocellular carcinoma cells (Fig. 4E).

Role of miR-9 in the inhibition of E-cadherin by PROX1

The role of miR-9 in PROX1-induced inhibition of E-cadherin was further studied. As mentioned above, 1-2 cells expressed very low level of PROX1 and miR-9 and high level of E-cadherin. Expression of pre-miR-9 precursor which targeted E-cadherin directly in 1-2 cells reduced E-cadherin protein dose-dependently (Fig. 5A). This is not a non-specific off-target effect because addition of a pre-miR negative control (at the concentration of 30 nmol/L) did

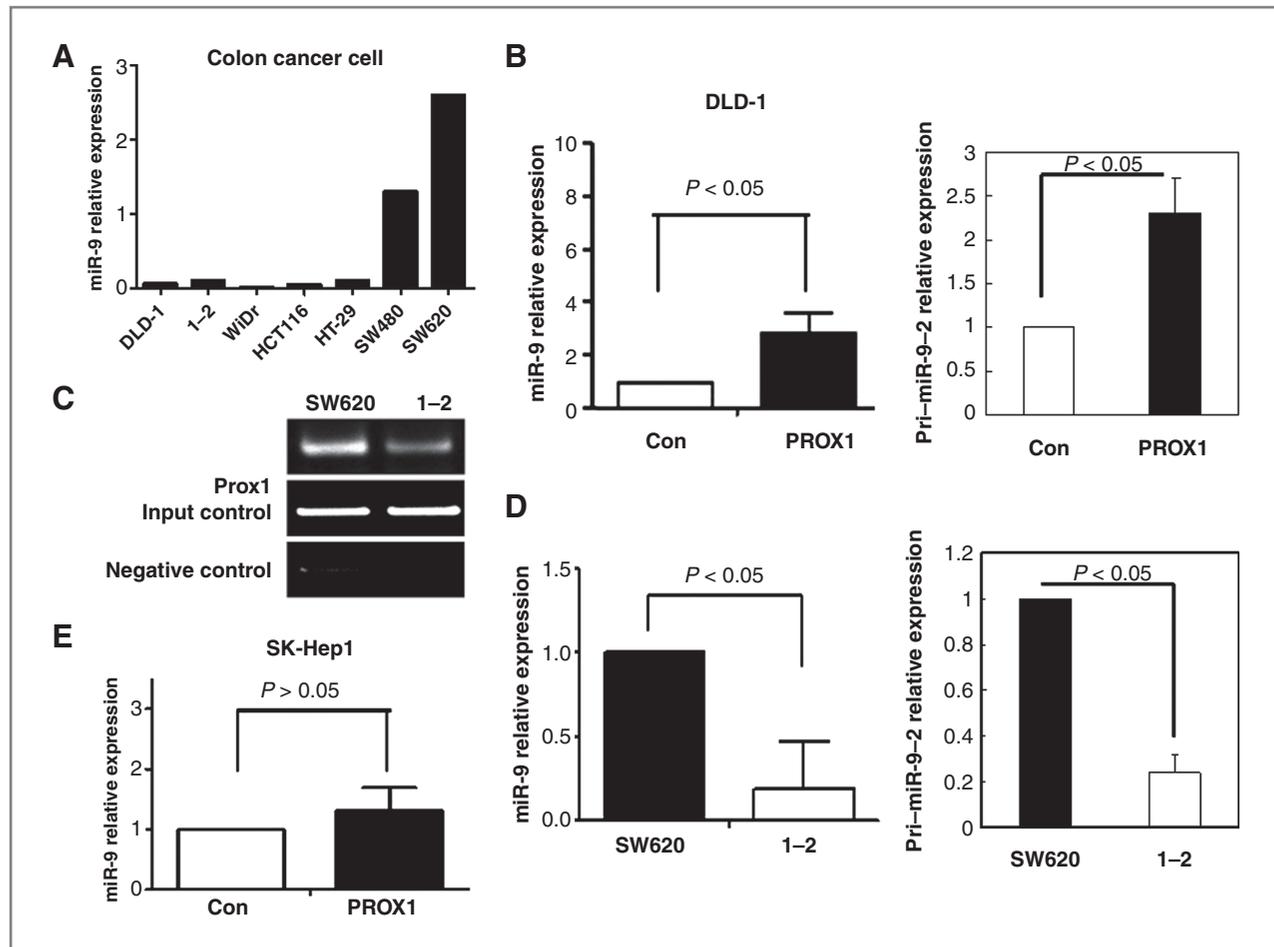


Figure 4. PROX1 binds to miR-9-2 promoter and upregulates its expression. **A**, expression of miR-9 in human colon cancer cell lines. **B**, PROX1 increased miR-9 and pri-miR-9-2 in colon cancer cell lines that expressed very low or undetectable endogenous PROX1. **C**, PROX1 bound to miR-9-2 promoter as shown by ChIP assay. This binding was significantly reduced in PROX1-knockdown 1-2 cells. **D**, expression of miR-9 and pri-miR-9-2 was much lower in 1-2 cells than that in SW620 cells. **E**, PROX1 did not affect miR-9 expression in SK-Hep-1 hepatoma cells indicating a cell type-specific regulation of miR-9 by PROX1.

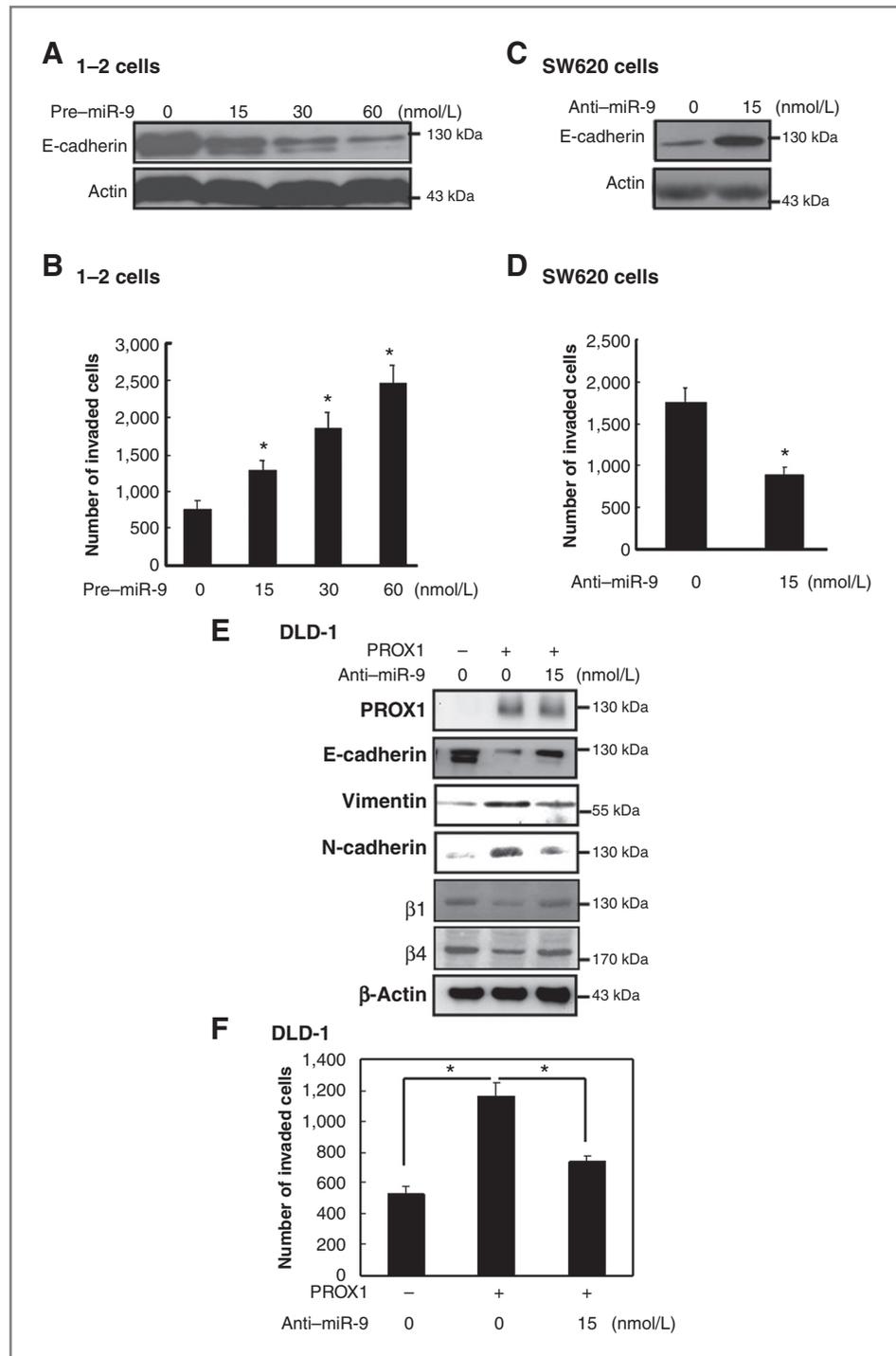
not affect E-cadherin protein level (Supplementary Fig. S5). In addition, the invasiveness of these cells was increased (Fig. 5B). Conversely, SW620 cells expressed high level of PROX1 and miR-9 and low level of E-cadherin. Transfection of anti-miR-9 increased E-cadherin level in these cells (Fig. 5C). As expected, invasive ability was attenuated after anti-miR-9 expression (Fig. 5D). These effects were not caused by cytotoxicity because anti-miR-9 did not affect cell viability (data not shown). The role of miR-9 in the inhibition of E-cadherin by PROX1 was further confirmed in DLD-1 cell. As shown in Fig. 5E, enforced expression of PROX1 led to reduction of E-cadherin and increase of vimentin and N-cadherin. Also, downregulation of integrin $\beta 1$ and $\beta 4$ was found. Coexpression of anti-miR-9 effectively reversed the increase of vimentin and N-cadherin and the reduction of E-cadherin and integrins. In addition, PROX1-induced increase of cell invasiveness was significantly suppressed by anti-miR-9 (Fig. 5F). These data suggested that miR-9 is important for the induction of EMT and cell invasion by PROX1. We tried to study the expression of miR-9 tumor

tissues with high PROX1/low E-cadherin or low PROX1/high E-cadherin from the same cohort used for IHC study. Only 7 samples were successfully isolated from these 2 groups separately because of the long-term storage (>10 years). However, our results showed that the mean miR-9 level of high PROX1/low E-cadherin group was higher (2.28-fold) than that of low PROX1/high E-cadherin group (Supplementary Fig. S6).

Discussion

The control of PROX1 gene transcription is tumor type-specific. For example, PROX1 has been shown to be a transcriptional target of β -catenin/TCF signaling pathway in colon cancer (12). However, this signaling pathway could not regulate PROX1 in liver cancer. Similarly, the functional role of PROX1 in carcinogenesis is dependent on cancer type. PROX1 functions as a tumor suppressor in breast cancer, liver cancer, and hematologic cancer (5-11). Conversely, PROX1 plays a tumor-promoting role in colon cancer, malignant astrocytic glioma, and kaposiform

Figure 5. miR-9 is required for PROX1-induced EMT and invasiveness. A, transfection of pre-miR-9 precursor reduced E-cadherin protein level dose dependently in PROX1-knockdown 1–2 cells. B, pre-miR-9 precursor increased invasive ability of 1–2 cells. *, $P < 0.05$. C, transfection of miR-9 inhibitor (anti-miR-9) restored E-cadherin protein level in SW620 cells. D, anti-miR-9 reduced invasive ability of SW620 cells. *, $P < 0.05$. E, DLD-1 cells were transfected with PROX1 expression vector or anti-miR-9 for 24 hours. Expression of PROX1, EMT-related markers, and integrins was studied by Western blotting. F, cells were also used for Transwell assay for the investigation of cell invasion. *, $P < 0.05$.



hemangioendothelioma (12–14). In this study, we provide evidence that PROX1 downregulates E-cadherin and promotes EMT in colon cancer cells. EMT is strongly associated with 3 important cancer characteristics: (1) metastasis, (2) cancer stem cell, and (3) drug resistance (24, 25). Our pathologic analysis clearly shows that PROX1 expression is linked with tumor stage and this transcription factor is not expressed in all of the tumors of Dukes stage A. In addition,

increase of PROX1 is significantly associated with lymph node metastasis. These data suggest that PROX1 upregulation promotes EMT and subsequently enhances metastasis in colon cancer. Interestingly, a recent study indicates that PROX1 may be involved in the regulation of cancer stem cells. By using an RNAi screening, Hope and colleagues show that PROX1 plays a key role in the control of hematopoietic stem cell activity (26). Our data support the notion

that PROX1 acts as a tumor promoter in colon cancer by enhancing EMT and metastasis.

We also show that PROX1 represses E-cadherin via miR-9. E-cadherin has been found to be generally inhibited by several EMT regulators such as SNAIL, TWIST, ZEB1, and ZEB2. However, we find that PROX1 does not control E-cadherin transcription (as verified by RT-PCR and promoter activity assays). Although 2 microRNAs (miR-31 and miR-181) have been shown to negatively regulate PROX1 expression in endothelial cells (27, 28), whether PROX1 could regulate the expression of microRNAs has never been reported. Our data show that PROX1 directly binds to *miR-9-2* promoter to upregulate its expression in colon cancer cells but not in liver cancer cells. The reason why PROX1 specifically activates miR-9 expression in colon cancer is not clear at present. We think this could be because of the complexity of miR-9 gene transcription. Intracellular amount of mature miR-9 is determined by miR-9-1, miR-9-2, and miR-9-3 genes that are located at different chromosomes. Because the expression of these 3 genes is controlled by different signaling pathways, it is obvious that a cell type-specific regulation will exist. For our study, we mainly focus on the miR-9-2 promoter. Regulation of miR-9-2 transcription by PROX1 is confirmed by (1) direct binding of PROX1 to miR-9-2 promoter and (2) the fold of increase of pri-miR-9-2 (the direct precursor transcript encoded by miR-9-2 gene) in PROX1 overexpression or knockdown cells is very similar to the change of mature miR-9 level. miR-9-2 is an intragenic microRNA and is positioned in the last exon of the annotated overlapping BC036480, AX746931, and CR599257 noncoding genes. Interesting, a recent study show that miR-9-2 is expressed independent of its host gene during human neuronal differentiation (29). It is possible that miR-9-2 expression can be controlled by the promoter of host gene or its own promoter in different cells. Study of this issue will be important for the elucidation of cell type-specific regulation of miR-9-2 by PROX1. Our cell-based assays also verify the importance of miR-9 in the downregulation of E-cadherin by PROX1. Moreover, we show that miR-9 level is significantly upregulated in high PROX1/low E-cadherin tumor tissues (Supplementary Fig. S6) although the sample size is limited because of the difficulty of miRNA isolation from tumor samples stored longer than 10 years. An extensive study with large sample number is warrant to confirm our conclusion.

Our data show a generally positive association between PROX1 and miR-9. For example, DLD-1 and WiDR cells do not express PROX1 and these cells express very low level of miR-9. Conversely, SW620 cells that express large amount of PROX1 also express high level of miR-9. However, it should be noted that different mechanisms for the control of miR-9 in different cell lines may exist. For instance, we find that SW480 cells that do not express PROX1 still express a significant amount of miR-9. A bioinformatics approach indicates that SW480 cells exhibit a gain of 5g14 chromosome region in which the miR-9-2 gene is located. There-

fore, the increase of miR-9 of some cancer cell lines may come from gene amplification. Induction of miR-9 has multiple advantages for PROX1 to promote cancer progression. In glioma, which PROX1 functions as a tumor promoter, miR-9 is also highly expressed with an unknown mechanism (30). MiR-9 represses a novel tumor suppressor calmodulin-binding transcription activator 1 (CAMTA1) that leads to the development of glioma stem cells (30). In addition, miR-9 inhibitor restores CAMTA1 expression, reduces neurosphere formation and attenuates tumor growth in nude mice. Because PROX1 has been shown to be highly expressed in glioma tissues and its expression is linked with high grade and poor outcome (14), it will be interesting to study the association of PROX1 and miR-9 in this cancer type to clarify whether miR-9 is also a critical mediator of PROX1 in glioma.

In addition to E-cadherin, we also show that increase of invasiveness of colon cancer cells by PROX1 can be mediated via other mechanisms including (1) modulation of integrin expression to alter cell adhesion and (2) enhancement of MMP expression and activity to promote matrix degradation. These data confirm the results of a previous study showing that PROX1 controls genes that involved in cell-cell and cell-matrix interactions (12). Integrins have been implicated in various cellular processes such as adhesion, migration, proliferation, and apoptosis (31–33). Therefore, PROX1 may modulate integrins to control cell motility and invasiveness. However, whether integrins are direct targets of PROX1 is still unknown. A very recent study suggests this possibility. The authors investigate the anti-inflammatory and anti-tumorigenic mechanism of estrogen receptor β in colon cancer (15) and find that this receptor may downregulate PROX1 expression. Their ChIP results show that PROX1 binds to promoter region of multiple integrin genes in various colon cancer cell lines. Interestingly, they find that integrin β 1 promoter is directly bound by PROX1 and its expression is reduced in PROX1-expressing HT-29 cells. This data is in consistent with our current results (Fig. 2B) showing that integrin β 1 is reduced by PROX1 in DLD-1 cells. We think integrins may be important mediators that participated in PROX1-induced colon cancer progression. Collectively, results of this study suggest that PROX1 increases invasiveness of colon cancer cells by modulating EMT, integrins, and MMPs.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: M.-H. Lu, M.-R. Pan, W.-C. Hung

Development of methodology: M.-H. Lu, M.-R. Pan

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): C.-C. Huang

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): M.-H. Lu, C.-C. Huang, M.-R. Pan, W.-C. Hung

Writing, review, and/or revision of the manuscript: C.-C. Huang, W.-C. Hung

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C.-C. Huang

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