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ORIGINAL RESEARCH

Prox1 Is Linked to Metastasis and Poor Prognosis by Promoting Lymphangiogenesis in Melanoma

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Purpose: The purpose of this study is to investigate the role of Prox1 in the progression of cutaneous melanoma (CMM) and its relationship with lymphatic metastasis.

Methods and Results: By analyzing the data from the Cancer Genome Atlas (TCGA), we found that the expression of Prox1 and LYVE1 was significantly upregulated in the metastatic melanoma group. Additionally, elevated levels of Prox1 were associated with shorter survival times. Correlation analysis demonstrated a significant relationship between Prox1 and markers associated with lymphangiogenesis, including LYVE1, FLT4, FOXC2, and ANGPT2. A clinical study involving 32 cases of CMM was conducted to analyze Prox1 expression and its relationship with lymphangiogenesis and clinicopathological characteristics. Research revealed that Prox1 was expressed significantly higher in patients with lymph node (LN) metastasis and in those classified as stage 3C-4. Additionally, the density of lymphatic vessels(LVD) in the LN metastasis group and the stage 3C-4 group was markedly higher than in the group without lymph node metastasis and in the stage 0–3B group. Furthermore, Breslow thickness was found to correlate with both Prox1 expression and LVD. Prox1-positive expression was associated with increased LVD. Further investigation was conducted on the role of Prox1 in the CMM cell line A375 and its derived exosomes. Exosomes were collected from CMM^{nc} and $CMM^{shProx1}$ to verify the changes in Prox1 expression, respectively. It was observed that the proliferation, migration, and tube formation abilities of human lymphatic endothelial cells(HLECs) diminished with the downregulation of Prox1. Additionally, VEGFR3 activation was reduced in HLECs following the reduction of Prox1.

Conclusion: Prox1 played an important role in promoting cell proliferation, migration, and lymphangiogenesis, which is related to tumor metastasis and poor prognosis. These results indicated the potential importance of Prox1 as a biomarker, which is expected to lead to the development of a new insight for anti-tumor therapy.

Keywords: melanoma, exosome, Prox1, lymphatic metastasis

Introduction

Over the past few decades, the application of immunotherapies and small-molecule inhibitors for melanoma has significantly improved the prognosis of a proportion of patients.[1,2] However, some problems remain to be solved, such as early lymphatic metastasis and the short survival time in advanced melanoma patients. Numerous studies have confirmed that LVD is positively correlated with the prognosis of patients,[3,4] and thin-layer melanoma also causes lymphatic metastasis.[5] Therefore, it is of great clinical significance to explore the mechanism of tumor-associated lymphangiogenesis and lymphatic metastasis in melanoma. Existing evidence identified that lots of factors can play an important role in promoting tumor-associated lymphangiogenesis and metastasis, for example, blocking the VEGFC/VEGFD-VEGFR3 signaling pathway can inhibit lymphangiogenesis and block tumor progression.[6–9] Prox1 was the master transcriptional switch for HLECs specification, lymphatic budding, and extension of HLECs,[10,11] and also associated with poor prognosis in a variety of tumors.[12,13]

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Experimental work has shown that lymphangiogenesis occurs in distant organs and sentinel lymph nodes (SLN) before the arrival of metastatic tumor cells, and was most likely stimulated by cytokines in body fluids drained from the primary tumor.[14,15] Exosomes are small microvesicles ranging from 30–150 nm in size that contain various types of proteins and nucleic acids. Many experiments have shown that tumor cell-derived exosomes can promote the early arrival of distant tissues, promote the formation of the tumor microenvironment, and promote tumor-associated lymphangiogenesis.[16–18] Exosomes, which are released by high-metastatic melanoma cells and carry miR-411-5p, activate the ERK pathway, ultimately boosting the proliferative colonization capacity of low-metastatic melanoma cells.[19] Research has revealed that exosomes from metastatic melanoma cells contain NGFR, and these exosomes could activate the ERK and NF-κB signaling pathways and upregulate ICAM-1 expression. These changes work together to boost lymphangiogenesis and facilitate the adhesion of melanoma cells.[7] But whether Prox1 acts via exosomes on HLECs is still unclear. The correlation between the expression level of Prox1 and lymphangiogenesis in melanoma has not been investigated.

Our findings highlight that Prox1 in melanoma cells and its-derived exosomes play an important role in regulating the tube formation ability and migration of HLECs and affect the invasion and metastasis behavior of melanoma, which shows its value for prognosis.

Materials and Methods

Patients Specimens

Paraffin-embedded tissue samples utilized in this study were obtained from 32 patients diagnosed with melanoma who underwent surgery between September 2015 and April 2021 at the Affiliated Hospital of Jiangsu University. Of these 32 cases, 25 specimens were sourced from the foot and toenails, which are classified as acral melanoma. Clinicopathological parameters were extracted from medical history records. All clinical procedures adhered to the Declaration of Helsinki and received approval from the Ethics Committee of our Affiliated Hospital of Jiangsu University. Furthermore, informed consent was obtained from each patient.

Bioinformatics Analysis

This study analyzes the differential expression of Prox1, LYVE1, and FLT4 across various cancers using the Tumor Immune Estimation Resource (TIMER 2.0) database (http://timer.cistrome.org/, accessed December 2023). The relationship between Prox1 mRNA expression and overall survival (OS) was assessed using the University of Alabama at Birmingham Cancer Data Analysis Portal (UALCAN) database (http://ualcan.path.uab.edu, accessed December 2022). Additionally, we investigated whether the Prox1 gene is associated with genes related to lymphatic vessels, utilizing TIMER 2.0 for this analysis.

Immunohistochemistry (IHC)

Paraffin-embedded sections of tumor tissues from patients were dewaxed and gradually rehydrated. Endogenous peroxidase activity was inhibited using a peroxidase-blocking solution. The sections were then incubated with monoclonal rabbit anti-human Prox1 (1:1000, Abcam) and LYVE1 (1:2000, Abcam) antibodies, respectively. Following incubation, the sections were stained using an HRP/DAB detection system and counterstained with Mayer's hematoxylin (Sigma–Aldrich, St. Louis, USA).

Evaluation of IHC

The IHC score of Prox1 was evaluated by two experienced pathologists who were unaware of the medical history, independently, as previously described.[20,21] The entire slide was examined at low magnification to identify the tumor's infiltration edge. Subsequently, five high-magnification (400x) fields were selected to calculate the percentage of positive cells, graded as previously outlined in CMM.[22] In summary, samples with 0–10% positive cells, 11%-50% positive cells, and more than 50% positive cells were classified as Prox1-negative, Prox1-medium, and Prox1-positive samples, respectively. Both Prox1-negative and Prox1-medium samples were considered to have low expression, while Prox1-positive samples were classified as having high expression.

Lymphatic vessels stained with the LYVE1 antibody were scanned at low magnification (40x), and fields of view exhibiting the highest concentrations of lymphatic vessels (hot spots) in the tumor or peritumoral parenchyma were selected. Hot spots were identified for each sample, and five hot spots were examined at high magnification (200x). LVD is defined as the mean number of lymphatic vessels from three hot spots.

Cell Culture

Human cutaneous melanoma cell line A375 was cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, USA) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (P/S) at 37°C in a 5% CO_2 atmosphere. The use of the cell line A375 had received approval from the Ethics Committee of our Affiliated Hospital of Jiangsu University.

HLECs were obtained from Shanghai JinYuan Biotechnology Co. (JinYuan, China) and cultured in endothelial cell culture medium (ECM) (ScienCell, USA) at 37°C with 5% CO_2. HLECs were utilized within six passages of culture.

All cell lines were routinely tested for mycoplasma and bacterial contamination.

Lentiviral Knockdown of Prox1

The lentivirus was customized and purchased from Genechem Co., Ltd., Shanghai. The multiplicity of infection (MOI) gradient was established according to the lentivirus instruction manual through preliminary experiments. In the formal experiment, lentiviruses (Lenti-control and Lenti-Prox1-shRNA) were transduced into A375 cells at an MOI of 50. The efficiency of transfection in the cells was evaluated using fluorescence intensity and Western blot analysis. Puromycin was used to eliminate uninfected melanoma cells.

Extraction of Exosomes Secreted by Melanoma Cells

Melanoma A375 cells were cultured in DMEM medium (Gibco) without serum for 72 hours prior to exosome extraction. A total of 500 mL of supernatant was collected and centrifuged at $10^{\wedge 4}$×g for 30 minutes to eliminate dead cells and cellular debris. Subsequently, the supernatant was concentrated using a centrifugal filter with a molecular weight cut-off (MWCO) of $10^{\wedge 5}$ Da, following the manufacturer's instructions (Millipore, USA). The ultrafiltered suspension was then passed through a 0.22 μm microporous membrane filter to remove any remaining cell debris and large vesicles. Exosomes were precipitated from the concentrates using the ExoQuick-TC extracellular vesicle (EV) isolation kit, adhering to the provided protocol (System Biosciences, USA). The final exosome sample was resuspended in PBS and stored at −80°C for future use.

Exosomes were collected from CMMs infected with an empty load virus (CMM^{nc}-Exo) and from CMMs transfected with a lentivirus to knock down Prox1 expression ($CMM^{shProx1}$-Exo).

Transmission Electron Microscopy Identification of Exosomes

The purified exosomes were appropriately diluted and placed onto a copper mesh for 5 minutes to allow for precipitation. Excess liquid was then absorbed using filter paper, and the sample was air-dried. Subsequently, a 3% solution of phosphotungstic acid in water was used to counterstain the sample for 2 minutes, after which the specimen was air-dried again. Finally, the exosomes were observed using transmission electron microscopy (Olympus, Japan) and photographed.

Western Blot

Whole-cell lysates or exosomes were harvested using RIPA buffer. Protein concentration was quantified with a BCA Protein Assay Kit (Vazyme Biotech, Nanjing, China), separated by 10% SDS-PAGE, and transferred onto a PVDF membrane (Millipore). The membranes were blocked with 5% nonfat milk powder in TBST for 1 hour and then incubated overnight at 4°C with primary antibodies [TSG101(1:2000, Abcam), CD9(1:2000, Abcam), CD63(1:2000, Abcam), Prox1(1:1000, Abcam), GAPDH(1:$20^{\wedge 4}$, Abcam), LYVE1 (1:2000, Abcam)]. Following this, the membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies for 1 hour at room temperature. Results were observed and visualized using an ECL detection system (Amersham Pharmacia Biotech, Little Chalfont, UK).

CCK-8

Cell proliferation was assessed using a CCK-8 assay (Vazyme Biotech, Nanjing, China). Cells were seeded at a density of $1 \times 10^{\wedge 3}$ cells per well in 96-well plates and cultured overnight at 37°C in a 5% CO_2 atmosphere. Cell proliferation was measured by the absorbance at 450 nm of reduced WST-8(2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-isulfophenyl)-2H-tetrazolium, monosodium salt) at the specified time points.

Transwell Migration Assay

In vitro, HLEC migration was examined using 24-well Transwell plates containing polycarbonate filter chambers with an 8-μm pore size (Corning Costar, Corning, NY, USA). HLECs cultured in 5% FBS were plated in the upper chamber of non-coated Transwell plates, while the lower chamber was filled with 10% FBS ECM medium. HLECs were co-cultured with CMM^{nc}-Exo and $CMM^{shProx1}$-Exo (50 μg/well) (n=4) for 16 hours. After incubation, the migrated cells that had passed through to the lower membrane were fixed with 4% buffered paraformaldehyde and stained with crystal violet. The number of migrated cells was quantified from the representative image.

Tube Formation

HLECs were co-cultured with CMM^{nc}-Exo and $CMM^{shProx1}$-Exo (50 μg/well) (n = 4) for 48 hours. They were then trypsinized, and $6 \times 10^{\wedge 4}$ HLECs were seeded onto Matrigel (BD Biosciences)-coated 96-well plates (10 μL/well). The degree of tube formation was photographed using an inverted microscope. HLEC tube formation was observed, and the normalized number of junctions formed by HLECs was calculated.

Statistical Analysis

All data were expressed as mean±standard deviation (SD), and two-tailed t-tests and one-way analysis of variance (ANOVA) were performed. The relationship between Prox1 expression and various clinicopathological parameters was assessed using the chi-square test and Fisher's exact test. The relationship between Prox1 expression and LVD was evaluated using Student's *t*-test. All statistical analyses were conducted using the Statistical Package for the Social Sciences (SPSS version 15.0; Chicago, IL, USA), with a significance level set at $p< 0.05$. Each experiment was repeated at least three times to obtain a P value and to control for systematic errors.

Results

The Differential Expression of Prox1 Is Positively Correlated with Poor Prognosis and Lymphatic Markers in Melanoma

Prox1 has been implicated in the progression of various types of cancer; however, its role in melanoma has not been thoroughly investigated. By analyzing RNA-seq data from multiple cancer types in TCGA, we found that Prox1 expression in 368 cases of metastatic melanoma was significantly higher than in 103 cases of melanoma in situ (Figure 1A–C). When patients were categorized based on Prox1 expression levels, survival analysis indicated that high Prox1 expression was associated with a shorter lifespan (Figure 1D). Additionally, the lymphatic endothelial cell marker LYVE1 exhibited higher expression in the metastatic melanoma group (Figure 1B and C). We observed a correlation between Prox1 expression levels and the lymphatic endothelial cell markers LYVE1, ANGPT2, FOXC2, and FLT4 in human melanoma (Figure 1E–H). Notably, these findings suggest that Prox1 is associated with poor prognosis and is linked to lymphatic involvement in melanoma patients.

Relationship Between Prox1, Clinicopathological Features and LVD

To further investigate the impact of Prox1 expression on the survival and prognosis of CMM patients, we conducted IHC to stain the target proteins Prox1 and the lymphatic endothelial cell marker LYVE1 in 32 surgical specimens. LVD was calculated as the mean number of lymphatic vessels from three hotspots in the adjacent tissues. We performed statistical analysis to assess the correlation between Prox1 expression and LVD in melanoma tissue. The IHC results revealed that

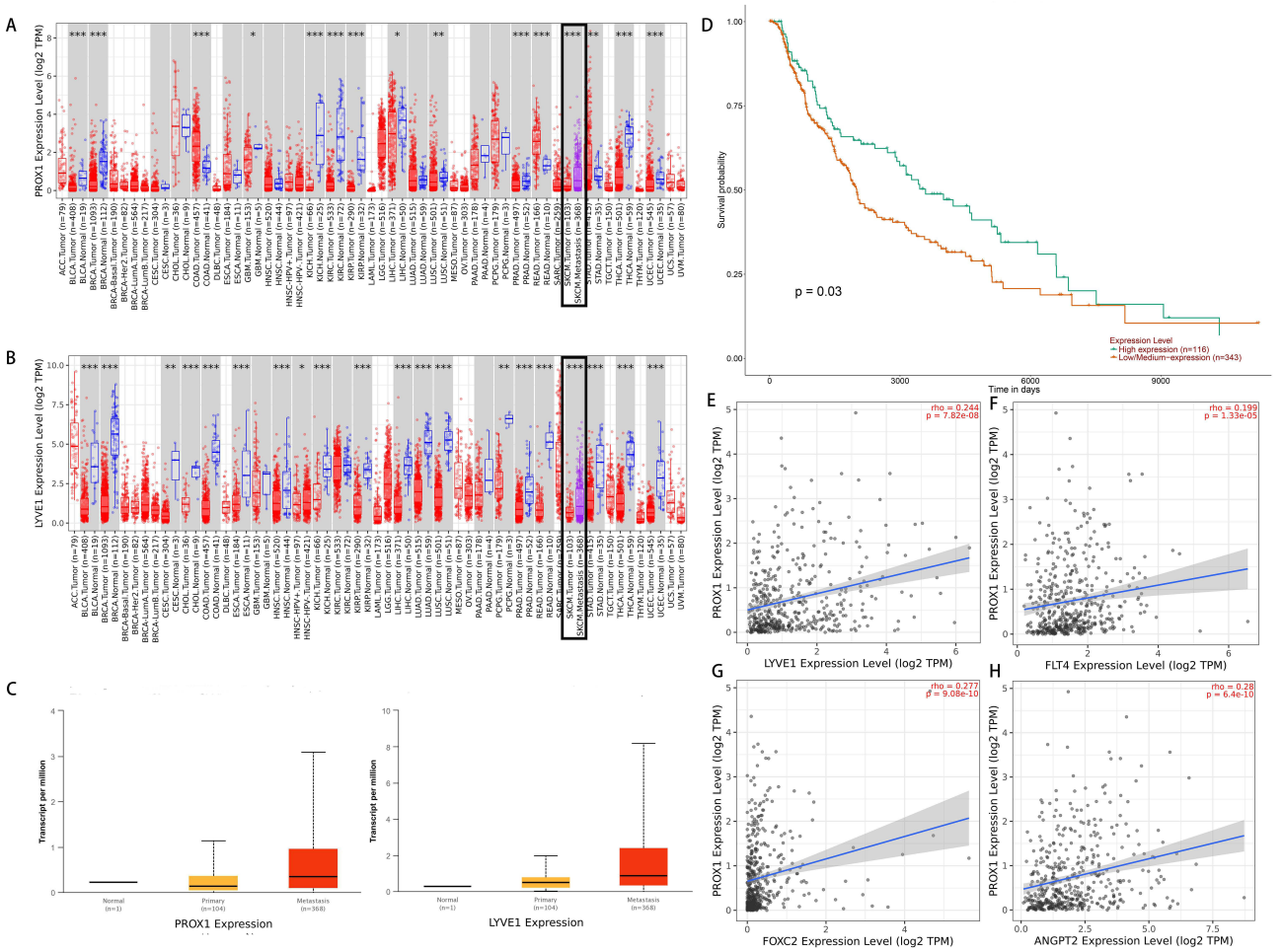

Figure 1 (**A**) Differential expression of Prox1 mRNA in various types of cancer, and the differential expression of Prox1 in primary and metastatic melanoma (black-colored rectangular box) was statistically significant. (**B**) Differential expression of LYVE1 mRNA in various types of cancer, and the differential expression of LYVE1 in primary and metastatic melanoma (black-colored rectangular box) was statistically significant. (**C**) Prox1/LYVE1 mRNA expression was significantly higher in the metastatic melanoma group compared to the primary group. (**D**) Prox1 mRNA expression is associated with the prognosis of patients with cutaneous malignant melanoma (CMM). (**E-H**) Prox1 expression levels positively correlate with the expression of lymphatic endothelial cell markers (LYVE-1, ANGPT2, FOXC2, FLT4) in CMM. *: $p < 0.05$; **: $p < 0.01$; ***: $p < 0.001$.

Prox1 was primarily expressed in the nucleoplasm of cancer cells. The number of lymphatic vessels in the group with high Prox1 expression was significantly greater than that in the group with low Prox1 expression (Figure 2).

The expression rate of Prox1 was significantly higher in the LN metastasis group, at 87.5%, compared to the non-metastasis group, which had a rate of 37.5% ($p < 0.05$). In the stage 3C-4 group, the proportion of cells positively expressing Prox1 was 79.17%, significantly exceeding the 12.5% observed in the stage 0–3B group ($p < 0.05$). Additionally, the positive expression rate for Prox1 was found to be 78.94% in the group with tumor thickness ≥ 2 mm, while it was only 38.46% in the group with tumor thickness < 2 mm ($p < 0.05$). In contrast, Prox1 expression did not show a significant correlation with age or gender ($p > 0.05$) (Table 1).

The LVD was measured at 8.88 ± 4.2 in the Prox1-high expression group and 5.15 ± 1.72 in the Prox1-low expression group, indicating significant differences between the two groups (Table 2). LVD is also closely associated with the patients' clinical characteristics as an independent indicator. In the lymph node metastasis group, the LVD was 9.30 ± 4.65, and in the stage 3C-4 group, it was 8.21 ± 4.19, both of which were significantly higher than the LVD in the non-lymph node metastasis group (5.66 ± 1.69) and the stage 0–3B group (5.30 ± 1.65), respectively, with all $p < 0.05$. Additionally, the mean LVD in the Breslow thickness ≥ 2 mm group (8.27 ± 3.87) was significantly higher than that in the Breslow thickness < 2 mm group (6.33 ± 3.81), with a $p < 0.05$ (Table 3).

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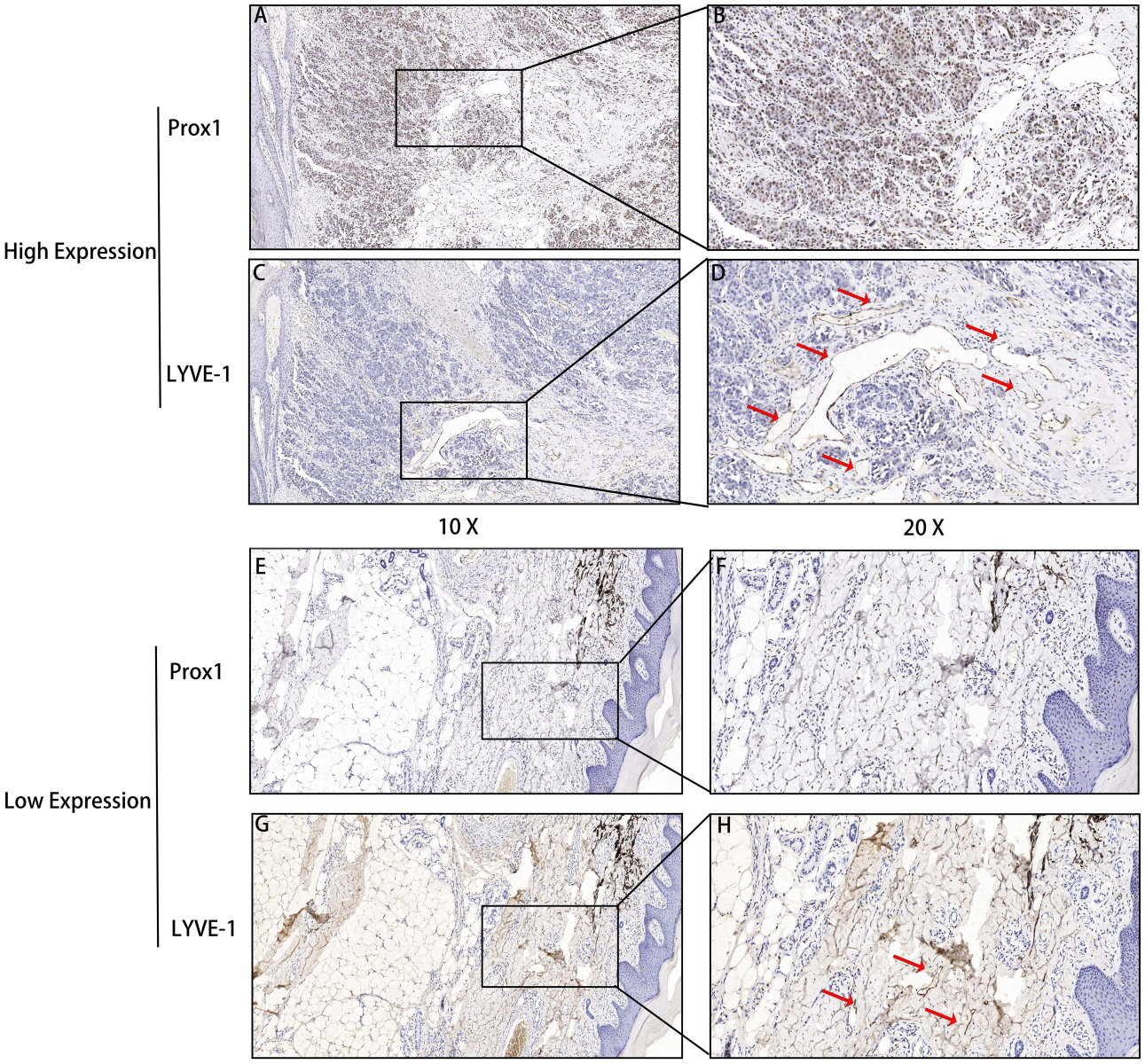

Figure 2 Prox1 expression and LYVE-1 labeled lymphatic vessels in CMM. (**A** and **B**) High Prox1 expression in tumor tissue (**A**) 10×, (**B**) 20×). (**C** and **D**) The number of lymphatic vessels was counted in areas of high Prox1 expression (**C**) 10×, (**D**) 20×). (**E** and **F**) Low Prox1 expression in tumor tissue (**E**) 10×, (**F**) 20×). (**G** and **H**) The number of lymphatic vessels was counted in areas of low Prox1 expression **G**) 10×, (**H**) 20×). The LVD was significantly higher in the Prox1 high expression group compared to the Prox1 low expression group. Red arrows indicate lymphatic vessels.

Prox1 Influences the Proliferation Function of CMM and Is Expressed on Exosomes Derived From CMM

To investigate the role of Prox1 in the progression of melanoma, we employed CRISPR-Cas9 gene editing to knock down Prox1 in melanoma cells. We verified the results and designated the Prox1 protein-deficient cell line as $CMM^{shProx1}$ (Figure 3B). We examined the impact of Prox1 on tumor proliferation. The results indicated that the proliferative capacity of melanoma cells was reduced following Prox1 knockdown (Figure 3G).

To further confirm that Prox1 not only influences the function of tumor cells but also impacts surrounding normal cells in the microenvironment through exosomes secreted by tumors, we analyzed bioinformatics results and clinical data. These findings indicate that Prox1 is closely associated with LVD and is significantly related to markers of

Table 1 Relationship Between Prox1 Expression and Clinical Characteristics in 32 Cases of CMM

Variable	N	High Expression (Cases, %)	χ^2	*P*
Age (years)				
<65	9	5 (55.56)	0.38	0.696
≥65	23	15 (65.22)		
Gender				
Male	18	14 (77.78)	1.81	0.068
Female	14	6 (42.86)		
LN metastasis				
Yes	16	14 (87.50)	8.53	0.009
No	16	6 (37.50)		
Clinical stage				
Stage 0–3B	8	1 (12.50)	11.38	0.002
Stage 3C-4	24	19 (79.17)		
Breslow thickness (mm)				
≥2	19	15 (78.94)	5.40	0.030
<2	13	5 (38.46)		

Abbreviations: N, number of specimens; LN, lymph node.

Table 2 Correlation Between Prox1 Expression and LVD

Prox1 Expression	N	LVD (±*SD*)	*r*	*p*
High	20	8.88±4.20	0.47	0.007
Low	12	5.15±1.72		

Abbreviations: LVD, lymphatic vessel density; SD, standard deviation; N, number of specimens; LN, lymph node.

Table 3 Relationship Between LVD and Clinical Characteristics of CMM

Variable	N	LVD±SD	*F/t*	*P*
Age (years)				
<65	9	7.45±4.37	0.086	0.772
≥65	23	7.87±4.30		
Gender				
Male	18	7.49±4.13	0.390	0.844
Female	14	7.48±3.75		
LN metastasis				
Yes	16	9.30±4.65	2.944	0.001
No	16	5.66±1.69		
Clinical stage				
Stage 0–3B	8	5.30±1.65	4.208	0.049
Stage 3C-4	24	8.21±4.19		
Breslow thickness (mm)				
≥2	19	8.27±3.87	4.551	0.046
<2	13	6.33±3.81		

Abbreviations: LVD, lymphatic vessel density; SD, standard deviation; N, number of specimens; LN, lymph node.

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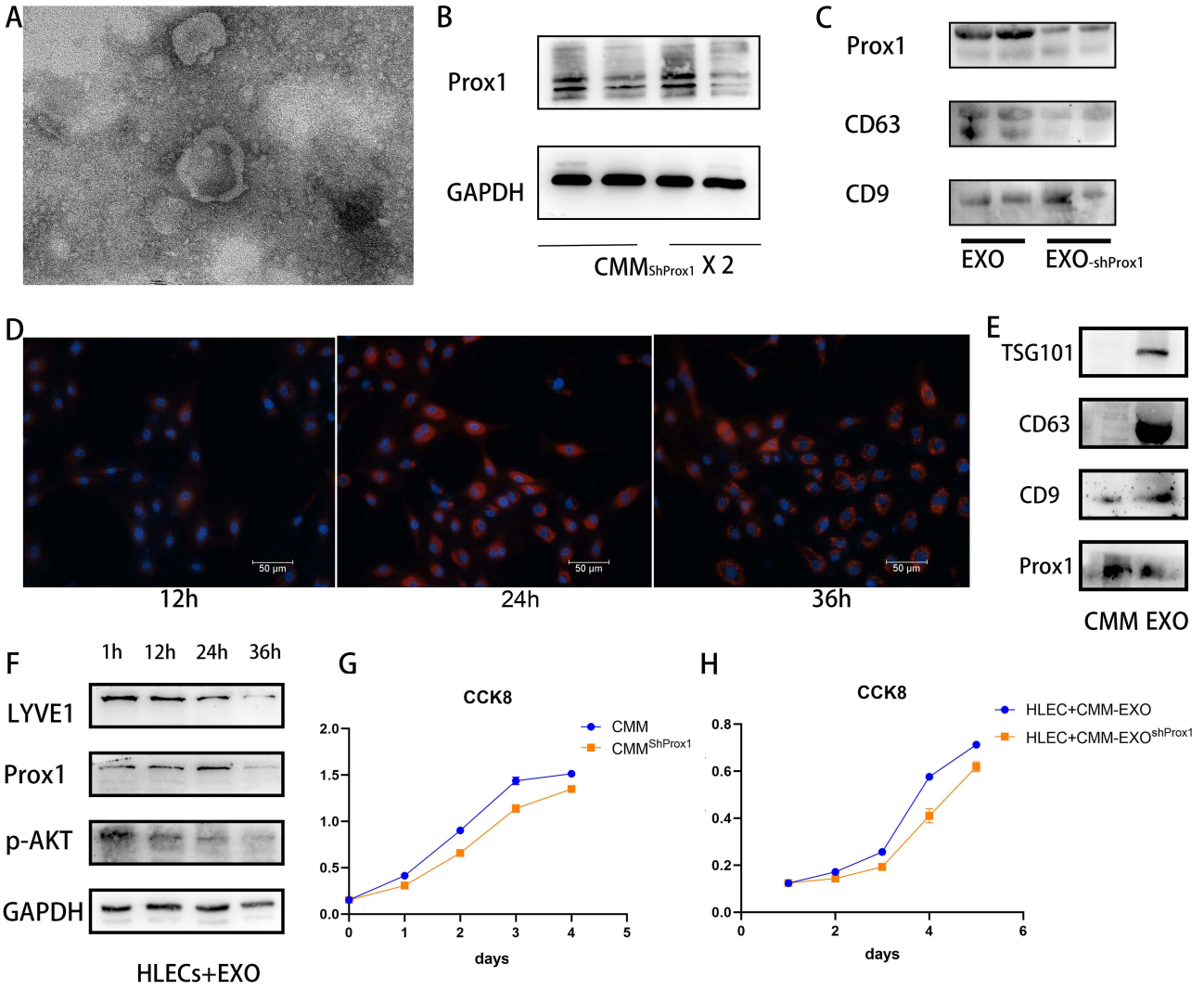

Figure 3 (**A**) Under the electron microscope, the exosomes appeared saucer-shaped and measured approximately 100 nm in diameter. (**B** and **C**) It was confirmed that Prox1 in CMMs had been successfully knocked down using lentivirus. (**D**) The dye-labeled exosomes were internalized by lymphatic endothelial cells. (**E**) Detection of exosome markers (CD9, CD63, TSG101) and the target protein Prox1 was performed using Western blot analysis. (**F**) Protein expression was changed after HLECs were co-cultured with melanoma exosomes. (**G**) Changes in cell proliferation ability in CMM after transfection with Prox1-shRNA (shProx1) were assessed using the CCK-8 assay. (**H**) Changes in cell proliferation ability in HLECs after HLECs co-culture are affected by Prox1 low expression.

lymphatic endothelial cells. Therefore, we speculate that Prox1 is packaged into tumor-derived exosomes and continuously taken up by lymphatic endothelial cells.

Subsequently, exosomes shed by CMM and CMM^{shProx1} were collected. The particle size of the melanoma exosomes was measured at 130 ± 10 nm using a transmission electron microscope (Figure 3A). This measurement is consistent with previous findings regarding the size of exosomes. Western blot analysis revealed that both CMM-Exo and CMM^{shProx1}-Exo expressed the exosomal tetraspanin markers CD63, TSG101, and CD9, as well as the target protein Prox1 (Figure 3C–E).

Prox1 Influences the Phenotype and Functional Changes of HLECs in vitro

Prox1 is a transcription factor that regulates the process of lymphangiogenesis. We hypothesized that exosomes are taken up by HLECs, and that this uptake activates part of the phenotype, leading to lymphangiogenesis and lymphatic metastasis in CMM, as indicated by previous studies. To investigate the impact of Prox1 derived from melanoma cells on HLECs, we co-cultured CMM-derived exosomes (CMM-Exo) and CMM^{shProx1}-derived exosomes (CMMshProx[1]-

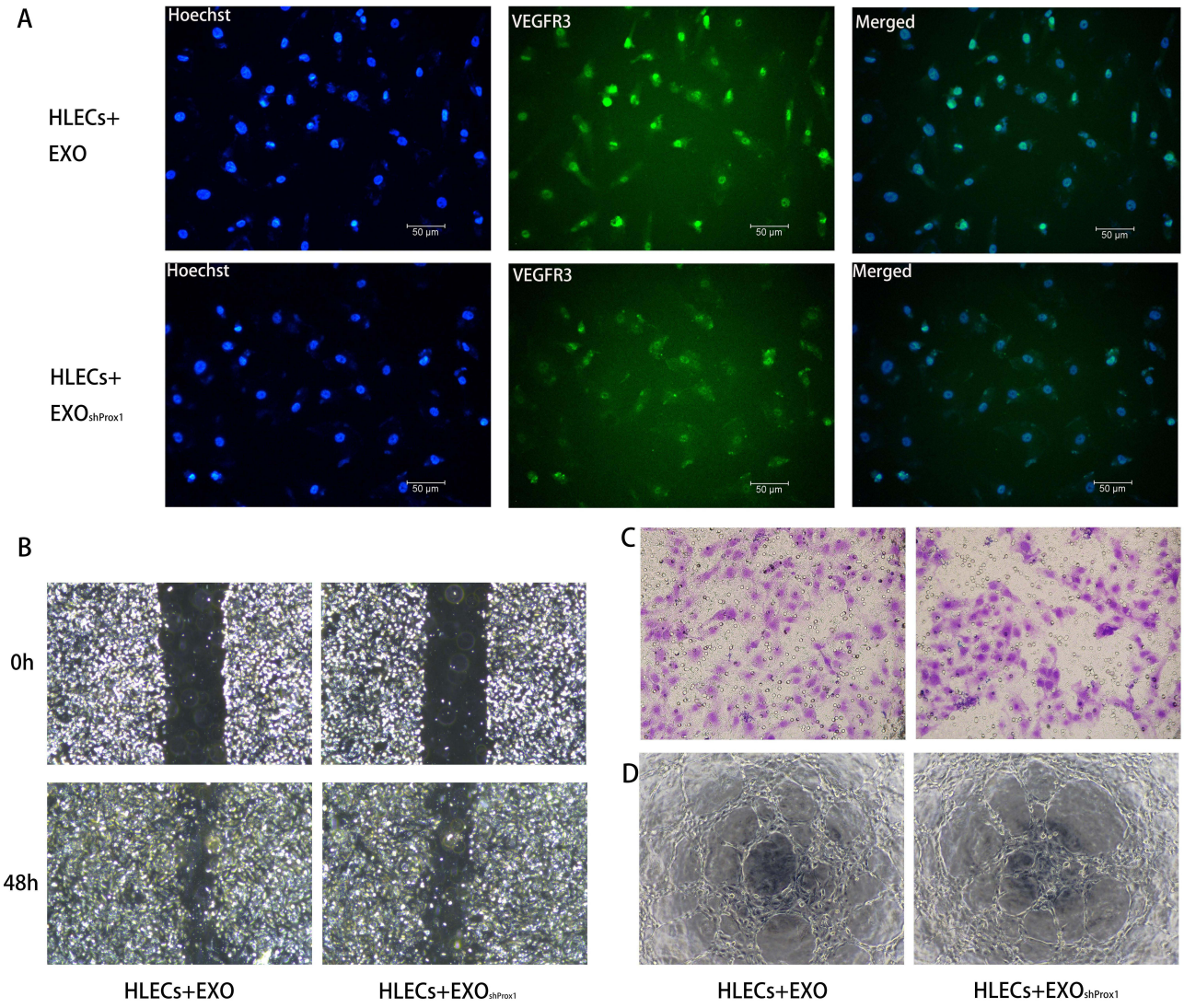

Figure 4 (**A**) When the expression of Prox1 in the exosomes derived from melanoma decreased, the fluorescent expression of VEGFR3 in the co-cultured HLECs weakened. (**B** and **C**) Affected by the reduction of Prox1 in melanoma exosomes, the migration ability of HLECs cells decreased after co-culture in Wound Healing Assay and Migration experiments. (**D**) The tube formation ability of the HLECs was reduced after co-culturing with $CMM^{shProx1}$-Exo.

Exo) with lymphatic endothelial cells, respectively. Confocal imaging results revealed that the labeled exosomes primarily localized in the cytoplasm and around the nucleus of HLECs after 12 hours of incubation. These findings suggest that HLECs can uptake exosomes in vitro (Figure 3D).

When Prox1 is altered in co-cultured conditions, it produces distinct effects on the phenotype and function of HLECs (Figure 3F). The activation of VEGFR3 was significantly lower in the $CMM^{shProx1}$-Exo co-culture group compared to the CMM-Exo co-culture group (Figure 4A). The proliferative and migratory capacity of HLECs was diminished in the $CMM^{shProx1}$-Exo co-culture group (Figures 3H, 4B and C). Additionally, the tube formation ability of HLECs co-cultured with $CMM^{shProx1}$-Exo was weaker than that of HLECs co-cultured with CMM-Exo (Figure 4D).

Discussion

Melanoma is highly prone to metastasis through lymphatic vessels, even in cases of thin melanoma. Acral melanoma was the most common type among the Asian population and was often diagnosed with a higher incidence of LN metastasis.[23] Numerous studies have reported a correlation between melanoma metastasis and lymphatic vessel density, indicating that lymphangiogenesis plays a crucial role in tumor LN metastasis.[14,24] It is crucial to investigate the core factors that

contribute to the increased number of microlymphatic vessels in the tumor microenvironment. Numerous studies have shown that before tumor metastasis, exosomes are released from tumors to LNs or the surrounding microenvironment to form pre-metastatic niches conducive to further metastasis.[25,26] In the premetastatic niche, capillaries and lymph capillaries are increased and more permeable, and the immune environment in the sentinel lymph nodes and around the tumor is also changed, resulting in a phenotypic change that is more favorable for tumor metastasis. Our clinical data confirmed that LVD and Prox1 levels in acral melanoma were significantly higher than those in normal tissues. The advanced melanoma group exhibited markedly elevated LVD and Prox1 levels compared to the in situ group. Statistical analysis revealed that LVD in the Prox1 high-expression group was significantly greater than that in the Prox1 low-expression group. An analysis of data from TCGA revealed significant differences in Prox1 expression across various cancers. High Prox1 expression in CMM was associated with a poor prognosis. Additionally, Prox1 was found to be highly expressed in metastatic melanoma and positively correlated with markers of lymphatic endothelial cells. However, the precise regulatory molecular mechanisms that promote tumor lymphangiogenesis require further investigation. Previous studies have indicated that Prox1 plays a role in tumor progression and has been linked to epithelial-mesenchymal transition (EMT) and oncogenic properties across various cancer types.[12,27,28] Furthermore, Prox1 expression has been shown to increase in breast cancer, correlating with tumor size and LN metastasis, and it can promote breast cancer invasion and metastasis.[12]

Exosomes have been widely studied for their role in intercellular communication between tumors and the tumor microenvironment. They are involved in processes such as proliferation, angiogenesis, lymphangiogenesis, and metastasis in various types of cancer.[29,30] Previous research has shown that exosomes secreted by bladder cancer cells promote lymphangiogenesis and lymph node metastasis in a VEGF-C-independent manner.[13] Additionally, exosomes derived from esophageal squamous carcinoma cells facilitate lymphangiogenesis by transferring circ_0026611.[31]

Another important finding in the present study was that Prox1 is highly expressed in CMM and CMM-Exo and was positively associated with lymphatic metastasis in patients. Our study demonstrates that CMM-Exo is a medium of crosstalk in tumor progression and lymphangiogenesis through intercellular communication with HLECs in a Prox1-dependent manner. Prox1 is packaged into melanoma cell-derived exosomes. Subsequently, exosomes are internalized by HLECs, resulting in the lymphangiogenesis and lymphatic metastasis of CMM. When Prox1 was knocked down by lentivirus in CMM cells, Prox1 was also reduced in expression in exosomes. When Prox1 low-expression exosomes were co-cultured with HLECs, the proliferation, migration, and tube formation ability of HLECs were reduced. We identified a novel pro-lymphangiogenesis factor, Prox1, which is enriched in CMM exosomes and plays an important role in CMM lymphatic metastasis. These findings provide new insights into the pathway by which exosome promotes melanoma lymphatic metastasis in a Prox1-independent manner, and support Prox1 as a novel therapeutic target in CMM.

It was identified that LN metastasis is strongly related to CMM mortality, and blocking this passage may be a promising treatment strategy for improving the prognosis of CMM. Our findings provided evidence that CMM-Exo may play an important regulatory role in tumor-associated lymphangiogenesis through the Prox1 signaling pathway, and Prox1 was over-expressed in melanoma cancer nest tissue and CMM-Exo, which positively correlated with LVD, and was clinically relevant to CMM LN metastasis. Our study not only identifies a crucial mechanism of exosomal Prox1-mediated intercellular communication from CMM to the HLECs to provoke LN metastasis, but also develops potential clinical applications in melanoma diagnosis of fluid, treatment, and prognosis assessment. Therefore, inhibiting the function of exosomal Prox1 might develop a new strategy for treating LN metastasis in CMM.

Conclusion

Prox1 has emerged as a central player in tumor progression, with its ability to drive cell proliferation, migration, and lymphangiogenesis directly contributing to tumor metastasis and poor patient prognosis. As research continues to unravel the molecular mechanisms underlying Prox1's actions - including its direct effect on melanoma cells and its role in the tumor microenvironment - we can expect to see the translation of Prox1-based biomarkers and therapies into clinical practice, ultimately improving outcomes for patients with aggressive, metastatic melanoma.

Abbreviations

CMM, cutaneous melanoma; TCGA, the Cancer Genome Atlas; LN, lymph node; LVD, the density of lymphatic vessels; HLECs, human lymphatic endothelial cells; SLN, sentinel lymph nodes; IHC, Immunohistochemistry; DMEM, Dulbecco's Modified Eagle's Medium; ECM, endothelial cell culture medium.

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Disclosure

The authors have declared that no competing interests exist in this paper.

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