

SOX7 blocks vasculogenic mimicry in oral squamous cell carcinoma

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Abstract

Background : Vasculogenic mimicry (VM) is the formation of an alternative circulatory system by aggressive tumor cells. The characteristics of VM and its underlying mechanism in oral squamous cell carcinoma (OSCC) remain unclear. This study aims to determine the relationship between VM channels in OSCC tissues and clinical outcomes and to investigate the biological role of SOX7 in VM in OSCC cells.

Methods : CD31/PAS double staining was performed to evaluate VM status in OSCC tissue. The relationships between VM and clinicopathological variables, and VM and SOX7 levels were analyzed. VM channel formation was assay performed to observe VM channels in the OSCC cell lines. To investigate the role of SOX7 in VM channel formation, SOX7 was transiently over-expressed in SCC-9 cells. VM-modulating genes were identified by Western blotting.

Results : We confirmed the presence of VM channels in OSCC tissue and several cell lines and found a positive correlation between VM and lymph node metastasis and patient survival in OSCC ($P = 0.003$). We also found that the presence of VM channels in OSCC tissue was inversely associated with SOX7 expression ($P = 0.020$). We observed that overexpression of SOX7 impaired VM channel formation by reducing the expression of VE-cadherin, thereby inhibiting cell migration and invasion.

Conclusion : These results suggest that SOX7 plays an important role in the regulation of VM channel formation and may inhibit OSCC metastasis.

Background

The formation of new blood vessels in solid tumors is essential for their growth and metastasis. Without the oxygen and nutrients provided by additional blood vessels, tumors undergo necrotic cell death due to ischemia and hypoxia [1, 2]. Conventional anti-angiogenic therapy, which targets vascular endothelial cells to inhibit tumor blood vessel formation, has proven unsatisfactory [2, 3]. The failure of such therapy suggests that tumors have an alternative strategy beyond angiogenesis for attaining a blood supply adequate to sustain growth.

A new interpretation of previous findings was reported by Maniotis et al., who observed highly patterned vessel-like channels in human melanomas in which red blood cells were detected, independent of endothelium-dependent angiogenesis [4]. Formed by the process of vasculogenic mimicry (VM), these structures consist of periodic acid–Schiff (PAS)⁺ and CD31-negative basement membranes lined by tumor cells rather than endothelial cells [5]. VM has been vividly described in numerous types of malignant tumors and is closely associated with a poor clinical outcome [6-8]. Experimental evidence has confirmed a relationship between VM and tumor metastasis in a variety of cancer cell lines and has shown that tumor cells can form VM channels in Matrigel culture [9-11]. The tumor cells lining VM networks express vascular endothelial cadherin (VE-cadherin) [12], erythropoietin-producing hepatocellular receptor A2 (EphA2) [13], and matrix metalloproteinases [14], the expression of which correlates with tumor metastasis and invasion [15]. Although many studies have contributed new insights into VM and its underlying molecular pathways [3, 16], the features of VM and its underlying mechanism in oral squamous cell carcinoma (OSCC) are still unclear.

We recently reported that the expression of SOX7, a member of the sex-determining region Y-box (SOX) family, is associated with advanced TMN stage, lymph node metastasis, and poor overall survival in patients with OSCC [17]. Additionally, several studies have reported a tumor-inhibitory function of SOX7 associated with cancer regulatory pathways [18, 19]. The effect of SOX7 on VM channel formation and the regulation of VM-related genes has not been studied in any cancer.

The present study investigates whether VM channels are present in human OSCC tissues and cell lines. The relationship between VM channels and clinical outcomes in OSCC tissues is examined, and the biological effect of SOX7 on VM channel formation in OSCC cells is explored.

Methods

Patients and tissue specimens

Specimens were retrieved from 46 patients with OSCC who were surgically treated at the Department of Oral and Maxillofacial Surgery at Seoul National University Dental Hospital between 1999 and 2005. Clinical data including age, sex, recurrence, and survival were collected from patient medical records. Tumors were graded based on the World Health Organization (WHO) Classification of Tumors [20] and staged according to the TNM system recommended by the American Joint Committee on Cancer [21]. The clinicopathological features of the OSCC patients are summarized in Table 1. This study was approved by the Institutional Review Board (IRB) of Seoul National University Dental Hospital (IRB number: ERI16008).

CD31/ Periodic acid–Schiff (PAS) double staining

Formalin-fixed, paraffin-embedded specimens were sectioned to a thickness of 4 μm . Tissue sections were deparaffinized in xylene and rehydrated in graded alcohol solutions. Endogenous peroxidase was inactivated by incubation in 3% H_2O_2 solution for 5 min. Heat-induced epitope retrieval was performed in citrate buffer (pH 6.0) for 10 min using a microwave oven. The sections were incubated overnight at 4°C with mouse monoclonal anti-CD31 (clone JC/70A; 1:100; Thermo Fisher Scientific, Rockford, IL, USA). The immunohistochemical reaction was performed using REAL EnVision/HRP (Dako, Glostrup, Denmark) and visualized by diaminobenzidine. Sections were incubated for 5 min in 0.5% periodic acid solution followed by 15 min in Schiff reagent. Slides were washed for 5 min in lukewarm water, counterstained with Mayer hematoxylin, dehydrated, and mounted. Endothelial cells were used as an internal positive control for CD31, and the primary antibody was omitted as the negative control.

Identification of vasculogenic mimicry

CD31/PAS double staining was evaluated blindly by two oral pathologists (KYO and SDH). Based on recent reviews of VM [22, 23], VM⁺ was defined as the presence of CD31⁻/PAS⁺ vessel-like structures surrounded by tumor cells on the non-luminal side. Although red blood cells may be observed in the lumen, the absence of endothelial cells was confirmed by the absence of CD31 expression on the luminal

side. Microscopic fields with prominent inflammatory infiltrates, necrosis, and hemorrhage were not included for VM evaluation. According to these criteria, all 46 OSCC cases were classified as VM⁺ or VM⁻.

SOX7 immunohistochemistry

Formalin-fixed, paraffin-embedded specimens were immunohistochemically stained using rabbit polyclonal anti-SOX7 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA) as previously described [17]. Immunohistochemical staining was assessed semi-quantitatively by two oral pathologists (KYO and SDH). Tumor cells showing nuclear staining were considered positive. The percentage of positive cells was scored as follows: 0, 0%; 1, 1–10%; 2, 11–50%; and 3, 51–100%. Staining intensity was scored as follows: 0, negative; 1, weak (light brown); 2, moderate (brown); and 3, strong (dark brown). A final score (0–9) was established by multiplying the percentage score by the intensity score. For statistical analysis, final scores ≥ 2 were defined as SOX7⁺, and scores < 2 as SOX7⁻.

Cell lines, Constructs, and Transfection

HN22, YD-10B, and OSC-20 cells were kindly provided by Dankook University (Cheonan, Korea), Yonsei University (Seoul, Korea), and Hokkaido University (Hokkaido, Japan), respectively. SCC-9 cells were obtained from American Tissue Culture Collection (Manassas, VA, USA). OSCC cell lines were cultured in DMEM/F-12 medium (Welgene, Daegu, Korea) containing 10% fetal bovine serum (FBS) and 100 U/mL penicillin/streptomycin in a humidified atmosphere containing 5% CO₂ at 37°C. Cells expressing pCMV6-Entry or pCMV6-SOX7 (RC210036, Origene, Rockville, USA) were transiently transfected with JET-PEI reagent (Polyplus-transfection, NY, USA).

Western Blot Analysis

Cell lysates were prepared by homogenization with RIPA buffer (EMD Millipore, Billerica, CA, USA) according to the protocol supplied. Protein concentrations were measured using the DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, USA). After normalization, equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to immunoblot polyvinylidene difluoride membranes (Pall Corporation, Port Washington, NY, USA). The membrane was blocked with 5% skim milk at room temperature for 2 h, incubated with specific primary antibodies, and probed with the corresponding horseradish peroxidase-conjugated secondary antibodies (GTX213110 for anti-Rabbit and GTX213111 for anti-mouse). Antibodies against SOX7 (SC-20093), VE-cadherin (SC-9989), and α -tubulin (SC-5286) were obtained from Santa Cruz Biotechnology. Rabbit anti-human monoclonal antibody against EphA2 (#6697) was obtained from Cell Signaling Technology, Inc (Danvers, MA, USA). The immunoreactive proteins were detected by SuperSignal West Pico Chemiluminescent Substrate (SC-2048; Santa Cruz Biotechnology), and immunoreactive bands were visualized using ImageQuant LAS 500 (GE Healthcare Life Sciences, Pittsburgh, PA, USA). Densitometric analysis of western blots was performed using ImageJ software (Version 1.51k, NIH, Bethesda, MD, USA).

Three-dimensional culturing for VM channel formation assay

A 24 well plate was coated with 150 μ L of growth factor reduced Matrigel (BD Bioscience, Bedford, MA, USA), and the cell suspension was seeded onto the solid Matrigel at a concentration of 1.5×10^5 cells with serum-free medium. After incubation for 24 h, cells were stained with PAS (Thermo Fisher Scientific) to label the basement membrane of tubular structures. Five random fields of view for channels and intersections were photographed at 100 \times magnification using an inverted microscope (Nikon Ti, Nikon Instruments Inc., Melville, USA) and counted using Image J software. Network Intersection was defined as a junction between 3 neighbors [24].

Cell migration and Invasion assays

To assess cell migration and invasion, cells (8×10^4 cells/well) were suspended in 0.5 mL of serum-free DMEM/F12 medium. For cell migration assays, collagen type I (BD Bioscience) was diluted in cold PBS solution and dried on the lower surface of transwell membranes (8- μ m pore size, Corning, Lowell, MA, USA). For invasion assays, the upper surface of the transwell membrane was pre-coated with a 1:15 dilution of growth factor reduced Matrigel (BD Bioscience). The bottom chamber was then filled with medium containing 10% FBS as a chemoattractant. The transwell chamber was incubated at 37 $^{\circ}$ C with 5% CO₂ for 24 h. Non-migrating and non-invading cells were removed from the top chamber with a cotton swab. Cells remaining on the lower surface were fixed with 100% methanol and stained with hematoxylin and eosin. The number of cells on the bottom chamber was counted under a light microscope (Leica DM5000B, Leica Microsystems, Wetzlar, Germany). For each assay, 10 different microscopic fields (100 \times magnification) were randomly selected.

Statistical analysis

Pearson chi-square test and Fisher's exact test were performed to evaluate the association between VM status and clinicopathological features or SOX7 expression. Cumulative survival curves were generated using the Kaplan–Meier method, and the difference between the curves was analyzed using the log-rank test. All *in vitro* data are expressed as the mean \pm standard deviation (SD). The statistical comparisons between groups were evaluated using Student's *t*-test for two groups. $P < 0.05$ was considered statistically significant. All statistical analyses were performed using IBM SPSS Statistics version 25 (IBM, Armonk, NY, USA).

Results

VM was associated with poor prognosis in OSCC patients

VM was identified in 23.9% (11/46) of the OSCC specimens studied. PAS⁺ tubular structures were lined by endothelial-like tumor cells with CD31⁻ staining (Figures 1A and B). Vascular-like structures were observed in 3 OSCC cell lines (HN22, OSC20, and YD10B), and PAS⁺ staining indicating VM channel formation was observed (Figure 1C). VM was significantly associated with lymph node metastasis ($P =$

0.003) (Table 2). The 5-year survival rate was 47.8% (22/46) in patients with OSCC, and the VM⁺ group tended to have lower overall survival ($P = 0.073$) (Figure 1D).

VM correlated with absence of SOX7 expression in OSCC

Of the 46 OSCC specimens, 14 (30.4%) were classified as SOX7⁺, and all were VM⁻ (Figure 2). All VM⁺ exhibited no nuclear staining for SOX7, indicating a strong association between the positive VM status and the absence of SOX7 expression in OSCC ($P = 0.020$) (Table 3).

SOX7 impaired VM channel formation in vitro

To assess the influence of SOX7 expression on VM channel formation *in vitro*, SCC-9 cells were transiently transfected with a SOX7 overexpression vector (Figures 3A and 3B). SOX7 and control (transfected with vehicle vector) cells were then cultured on Matrigel for 24 h. SCC-9 cells transfected with SOX7 had fewer channels than did control cells and did not form extensive channel networks (Figure 3C). These results were confirmed by PAS staining of VM networks (Figure 3C) and quantification of channel formation and the number of channel intersections indicated a decrease in VM channel formation in SOX7-overexpressing cells (Figure 3D). VE-cadherin and EphA2 have been identified as major proteins involved in VM channel formation [12]. While the expression of EphA2 did not change significantly, we found that only VE-cadherin expression was reduced in SOX7-overexpressing cells (Figures 3E and 3F).

SOX7 inhibited the migration and invasion of SCC-9 cells

VM channel formation is thought to be related to tumor cell migration and invasion [5]. To investigate the function of SOX7 expression in the migration and invasion ability of SCC-9 cells, we performed transwell migration and invasion assays in SCC-9 cells. As expected, SOX7 overexpression strongly inhibited the migration and invasion capacity of SCC-9 cells (Figure 4).

Discussion

Avascular tumors (>1–2 mm³) cannot be sufficiently supplied with oxygen and nutrients and cannot rely entirely on host endothelial cells for angiogenesis [25]. Therefore, tumors require a system independent of angiogenesis, such as VM. A three-stage phenomenon underlies VM: 1) highly malignant tumor cell plasticity, 2) remodeling of the extracellular matrix (ECM), and 3) connection of VM channels and host blood vessels to obtain a blood supply from the host tissue [26]. Since the first description of VM in uveal melanoma, a CD31⁻/PAS⁺ profile has been recognized as the hallmark of VM [10, 24, 27]. In this study, we observed small vessel-like structures with a lumen lined with tumor cells in OSCC tissue (Figures 1A and 1B). VM channels were also detected in several OSCC cell lines using Matrigel culture system (Figure 1C). In addition, the occurrence of VM was positively associated with lymph node metastasis (Table 2), and Kaplan–Meier survival analysis showed that VM⁺ OSCC patients had a worse overall survival rate than did VM⁻ patients (Figure 1D). Our present findings are consistent with a recent meta-analysis reporting that VM is associated with lymph node metastasis and overall survival in patients with squamous cell

carcinoma of the head and neck [28]. These observations strongly suggest that VM may be used as an independent predictor of prognosis in OSCC.

We previously demonstrated that SOX7 expression was lower in OSCC tissue samples than in corresponding normal oral mucosal tissues and that the absence of SOX7 expression was significantly associated with advanced TNM stage, incidence of lymph node metastasis, and poor prognosis [17]. SOX7 expression was found to be frequently down-regulated in a variety of tumors; its forced expression inhibited cellular proliferation, migration, and invasion, suggesting that SOX7 functions as a tumor suppressor [29-31]. Because both VM and SOX7 are closely associated with OSCC prognosis, we hypothesized that SOX7 might play an essential role in VM channel formation in OSCC. In the present study, we observed VM channels in 34.4% of OSCC in the absence of SOX7 expression; SOX7 expression correlated negatively with VM status (Figure 2 and Table 3). *In vitro* experiments also showed that overexpression of SOX7 severely disrupted the formation of VM channels in SCC-9 cells in a Matrigel culture system (Figure 3C and 3D). These results further support the hypothesis that SOX7 regulates VM channel formation in OSCC. To the best of our knowledge, this is the first report elucidating the role of SOX7 in VM in OSCC.

Given that tumor cells can be transformed into endothelial-like cells (mesenchymal cells), many studies have proposed that the epithelial–mesenchymal transition (EMT) is vital for VM [32-35]. A recent study reports that the EMT-related transcription factors Twist1 and Snail were up-regulated in VM⁺ hepatocellular carcinoma samples and that their expression correlated with VM channel formation [33, 36]. In contrast, our results show that VM status was not associated with the expression of Snail or Twist in OSCC (data not shown), indicating that other factors are involved in VM in OSCC. VM-associated molecules and signaling pathways have been investigated in numerous types of highly aggressive malignant tumors and have served as a strategic roadmap for drug development [15]. VE-cadherin, a transmembrane protein in the cadherin family, is expressed exclusively by endothelial cells [37]. Expressed in highly aggressive melanoma cells, its down-regulation was observed to abolish VM, indicating its involvement in VM structure formation [38]. VE-cadherin also mediates the activities of EphA2, a member of the ephrin-receptor family of PTKs, which is crucial for angiogenesis [39]. Mitra et al., recently found that phosphorylation of EphA2 was significantly associated with the occurrence of VM in invasive breast carcinoma [40]. Co-localization of VE-cadherin with EphA2 is reported to induce the activation of MMPs, resulting in matrix remodeling and VM channel formation [12, 15, 41]. These findings suggest that VE-cadherin, EphA2, and MMPs are important VM-modulating genes. In this study, we observed that SOX7 overexpression significantly attenuated the protein expression of VE-cadherin in SCC-9 cells but did not affect VM-modulating genes such as EphA2 and MMP-2 and -9 (Figure 2E; Supplementary Figure 1). These results suggest that the molecule responsible for the observed regulatory effects of SOX7 on VM channel formation in OSCC is VE-cadherin.

Conclusion

Our findings show that the occurrence of VM correlated positively with lymph node metastasis and may be a promising marker of patient prognosis in OSCC. We also provide evidence that the presence of VM channels in OSCC tissue is inversely associated with SOX7 expression and that SOX7 restrains *in vitro* VM channel formation by inhibiting VE-cadherin expression. To the best of our knowledge, this study is the first to demonstrate that SOX7 plays an important role in VM and consequently contributes to OSCC suppression. Therefore, further investigation of SOX7 as a potential therapeutic strategy for inhibiting VM channel formation in OSCC is necessary in the future.

Abbreviation

SOX7: Sex-determining region Y-box (SOX) family; VM: Vasculogenic mimicry; OSCC: Oral squamous cell carcinoma; PAS: Periodic acid–Schiff; VE-cadherin: Vascular endothelial cadherin; EphA2: Erythropoietin-producing hepatocellular receptor A2; PTK: Protein tyrosine kinases; EMT: Epithelial–mesenchymal transition; MMPs: Matrix metalloproteinases.

Declarations

Ethics approval and consent to participate

This study was approved by the SNU DH (Seoul National University Dental Hospital) Institutional Review Board.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

Funding

Not applicable.

Authors' contribution

Study concepts: SDH, KOH; Study design: KYO, HJY, SDC; Data acquisition: KOH, KYO, NS; Quality control of data and algorithms: MJ, JHK, JAS; Data analysis and interpretation: KOH, KYO, SDH; Statistical

analysis: NS, JIL, MJ, JHK; Manuscript preparation: HJY, JAS, KYO; Manuscript editing: SDC, KOH; Manuscript review: SDC, SDH. All authors read and approved the final manuscript.

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Tables

Table 1. Clinicopathological features of 46 OSCC patients

Variable	No. of cases (%)
Age (y); mean, 55	
20–29	2 (4)
30–39	2 (4)
40–49	11 (24)
50–59	15 (33)
60–69	11 (24)
70–79	5 (11)
Sex	
Male	40 (87)
Female	6 (13)
Differentiation status	
Well	34 (74)
Moderately	12 (26)
T classification	
T1	6 (13)
T2	24 (52)
T3	2 (4)
T4	14 (30)
N classification	
N0	17 (37)
N1	12 (26)
N2	17 (37)
M classification	
M0	45 (98)
M1	1 (2)
Stage	

I	4 (9)
II	5 (11)
III	10 (22)
IV	27 (59)
Recurrence	
No	34 (74)
Yes	12 (26)

Table 2. Associations between vasculogenic mimicry status and clinicopathological features in patients with OSCC

	No. of patients (n = 46)	Vasculogenic mimicry status		<i>P</i>
		Negative (n = 35)	Positive (n = 11)	
Age (y)				
<55	21	16	5	0.988
≥55	25	19	6	
Gender				
Male	40	30	10	1.000
Female	6	5	1	
Differentiation status				
Well	34	27	7	0.441
Moderately	12	8	4	
Tumor size				
T1+T2+T3	32	22	10	0.133
T4	14	13	1	
Lymph node metastasis				
Negative	17	17	0	0.003*
Positive	29	18	11	
Distant metastasis				
Negative	45	34	11	1.000
Positive	1	1	0	
Stage				
I + II + III	19	17	2	0.092
IV	27	18	9	
Recurrence				
No	34	25	9	0.701
Yes	12	10	2	

* $P < 0.05$ vs control group

Table 3. Correlation between vasculogenic mimicry and SOX7 expression in OSCC

	No. of patients (n = 46)	Vasculogenic mimicry status		<i>P</i>
		Negative (n = 35)	Positive (n = 11)	
SOX7				
Negative	32	21	11	0.020*
Positive	14	14	0	

* $P < 0.05$ vs control group

Figures

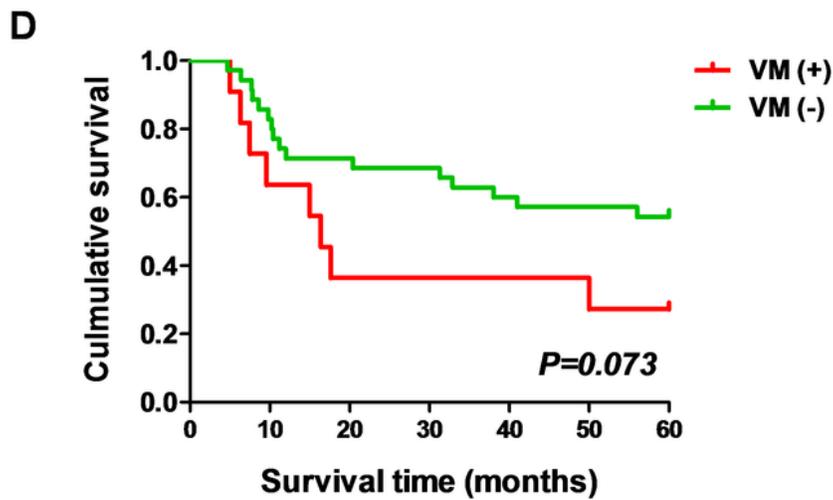
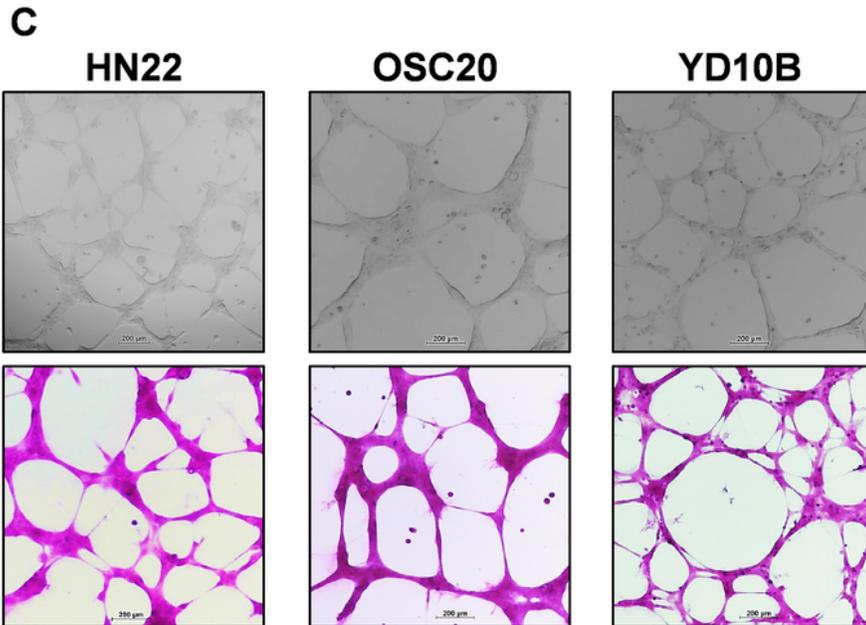
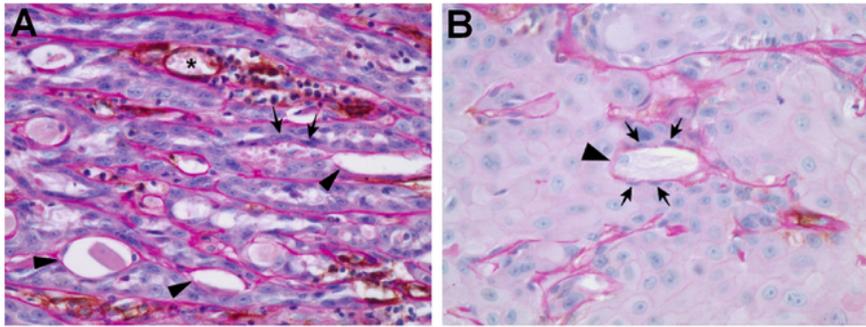


Figure 1

Vasculogenic mimicry (VM) in OSCC. (A and B) Representative images of CD31/PAS double staining in OSCC tissue sections. The arrowheads show CD31⁻/PAS⁺ tubular structures in a VM⁺ case. A vessel-like structure surrounded by tumor cells (arrows) contains red blood cells. Asterisk shows a blood vessel lined by CD31⁺ endothelial cells (original magnification, 400 \times). (C) Phase-contrast microscope images of channel formation were taken from various OSCC cell lines (top panel), and PAS-stained images of OSCC

cell lines (bottom panel) (original magnification, 100×). (D) Kaplan–Meier survival curves of OSCC patients. VM+ patients showed a tendency toward poorer overall survival (P = 0.073).

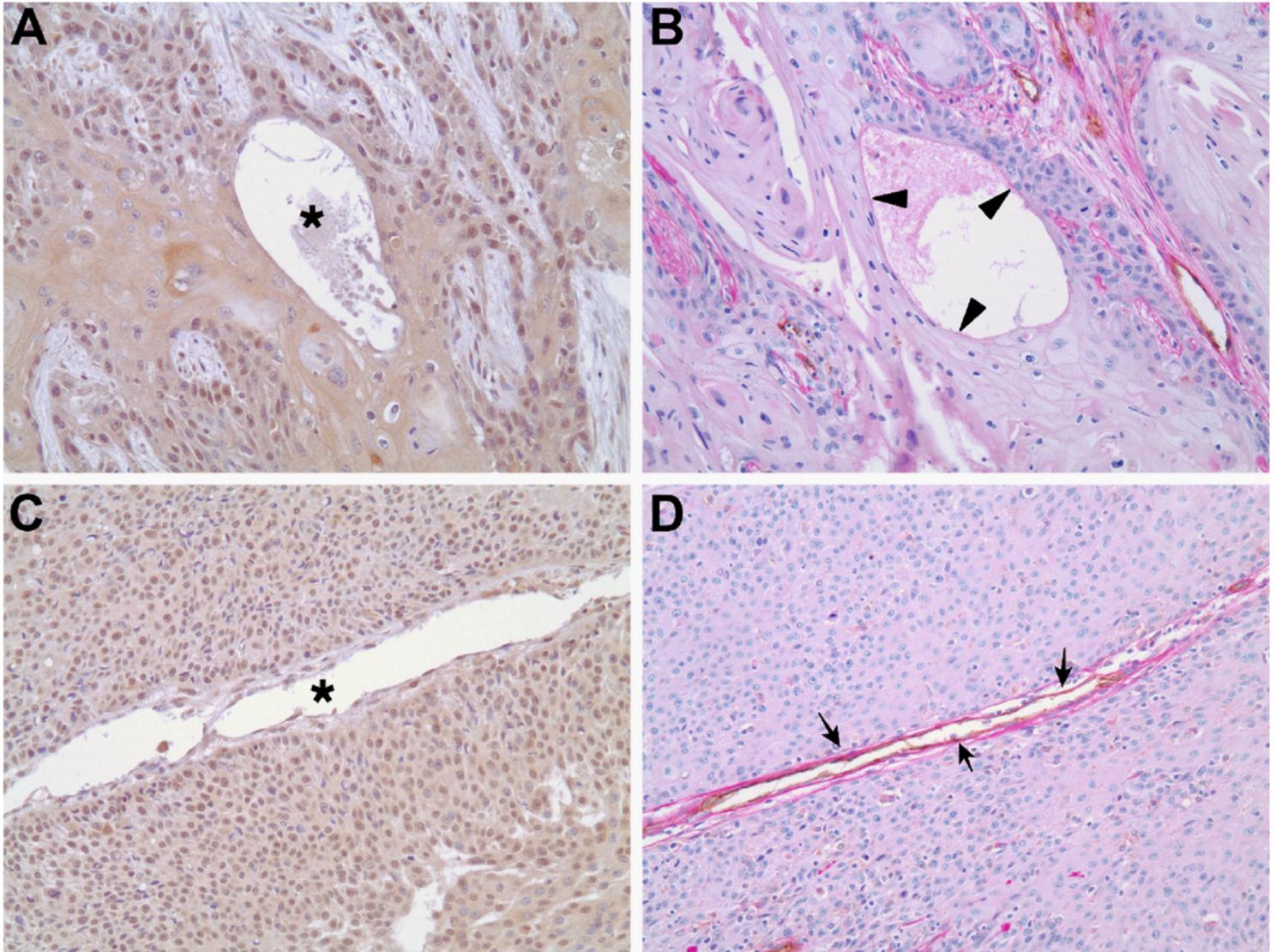


Figure 2

SOX7 immunohistochemical (A and C) and CD31/PAS double staining (B and D) in OSCC (original magnification, 200×). (A and B) A case showing diffuse nuclear staining of SOX7 has a lumen (asterisk) suggesting VM. However, the luminal side was negative for PAS staining (arrowheads). (C–D) Another SOX7+ case shows vasculature (asterisk) lined by CD31+ endothelial cells (arrows).

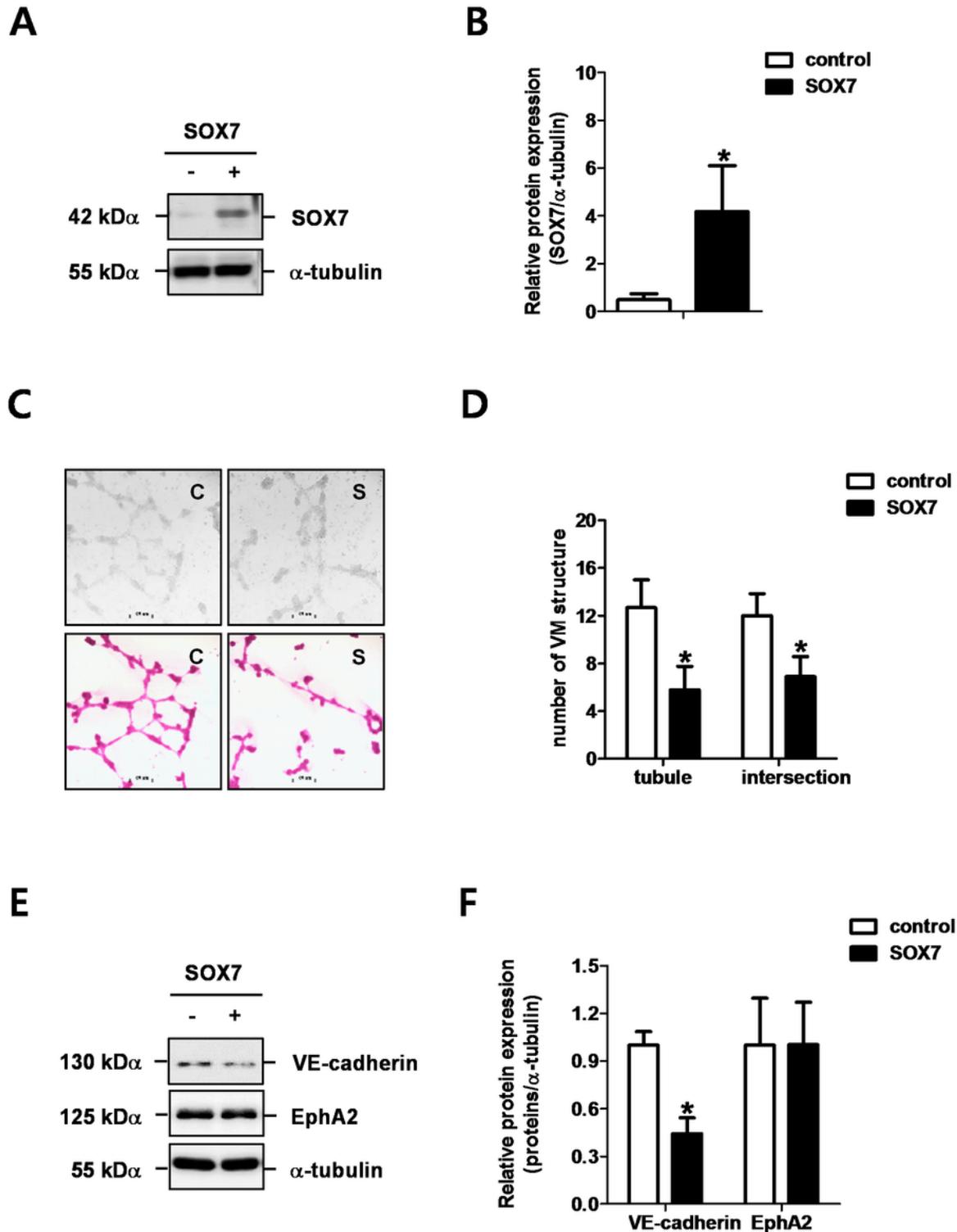


Figure 3

Inhibitory effect of SOX7 on VM channel formation in SCC-9 cells. (A and B) SOX7 expression in SCC-9 cells after transfection with pCMV6-SOX7 vector (SOX7 [S]) or pCMV6-empty (control [C]) as assessed by Western blot analysis. (C) phase-contrast microscopy images of channel formation in SCC-9 cells (upper row); PAS-stained images of SCC-9 cells grown on Matrigel for 24 h (lower row) (original magnification, 100 \times). (D) For quantitative analysis, five non-overlapping fields were selected from each culture well;

graph shows the mean \pm SD of three independent experiments. *P < 0.05 vs control group. (E) Western blot analysis assessing the effect of exogenous SOX7 on the expression of VM-modulated proteins (VE-cadherin and EphA2) in SCC-9 cells. (F) Graph shows the mean \pm SD of three independent experiments. *P < 0.05 vs control group.

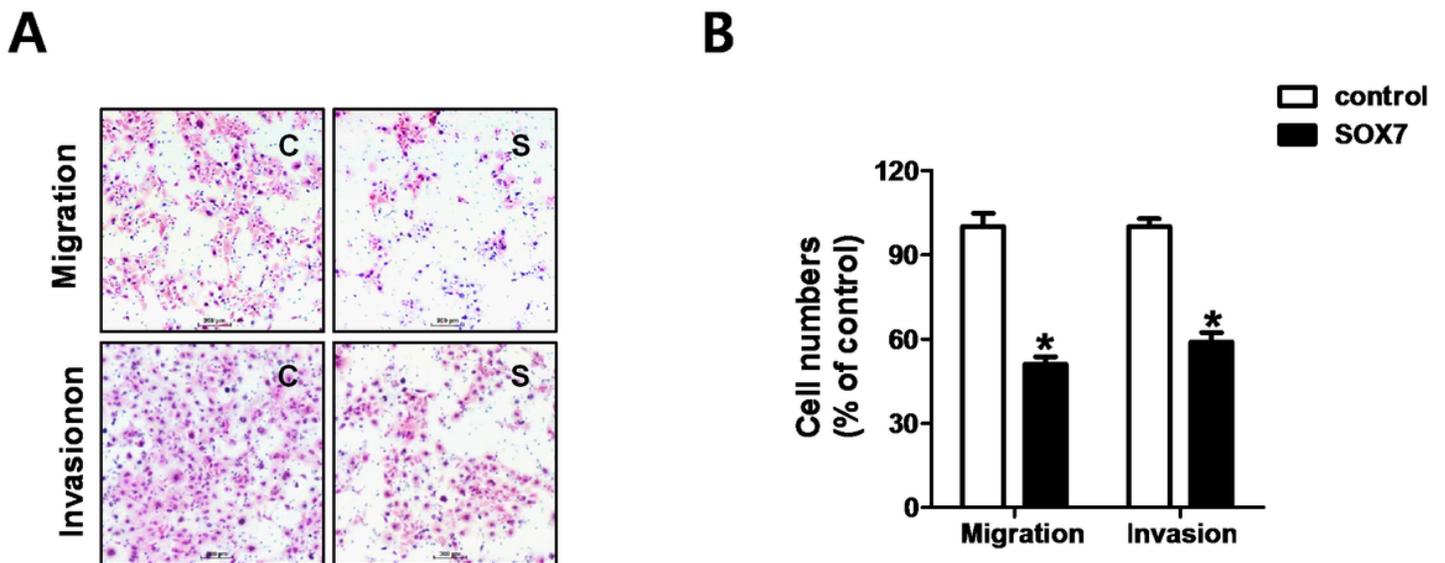


Figure 4

Migration and Matrigel invasion assay of SCC-9 cells (A) Cells overexpressing SOX7 exhibited impaired migration and invasion. (B) For quantitative analysis, five non-overlapping fields were selected from each culture well; graph shows the mean \pm SD of three independent experiments. *P < 0.05 vs control group.

Supplementary Files

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