Tumor and Stem Cell Biology

SIX1 Promotes Tumor Lymphangiogenesis by Coordinating TGFβ Signals That Increase Expression of VEGF-C

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Abstract

Lymphatic vessels are one of the major routes for the dissemination of cancer cells. Malignant tumors release growth factors such as VEGF-C to induce lymphangiogenesis, thereby promoting lymph node metastasis. Here, we report that sine oculis homeobox homolog 1 (SIX1), expressed in tumor cells, can promote tumor lymphangiogenesis and lymph node metastasis by coordinating with TGFβ to increase the expression of VEGF-C. Lymphangiogenesis and lymph node metastasis in cervical cancer were closely correlated with higher expression of SIX1 in tumor cells. By enhancing VEGF-C expression in tumor cells, SIX1 could augment the promoting effect of tumor cells on the migration and tube formation of lymphatic endothelial cells (LEC) in vitro and lymphangiogenesis in vivo. SIX1 enhanced TGFβ-induced activation of SMAD2/3 and coordinated with the SMAD pathway to modulate VEGF-C expression. Together, SIX1 and TGFβ induced much higher expression of VEGF-C in tumor cells than each of them alone. Despite its effect in promoting VEGF-C expression, TGFβ could inhibit lymphangiogenesis by directly inhibiting tube formation by LECs. However, the increased production of VEGF-C not only directly promoted migration and tube formation of LECs but also thwarted the inhibitory effect of TGFβ on LECs. That is, tumor cells that expressed high levels of SIX1 could promote lymphangiogenesis and counteract the negative effects of TGFβ on lymphangiogenesis by increasing the expression of VEGF-C. These findings provide new insights into tumor lymphangiogenesis and the various roles of TGFβ signaling in tumor regulation. Our results also suggest that SIX1/TGFβ might be a potential therapeutic target for preventing lymph node metastasis of tumor. Cancer Res; 74(19); 5597–607. ©2014 AACR.

Introduction

Tumor-induced lymphangiogenesis is correlated and functionally associated with tumor metastasis and poor prognosis (1, 2). Similar to tumor angiogenesis, lymphangiogenesis is mainly influenced by the tumor microenvironment (3). Among a variety of factors, VEGF-C and VEGF-D are considered the key drivers of lymphangiogenesis (4–8). VEGF-C is essential for lymph node metastasis of tumor. SIX1, a regulatory homeoprotein that plays a crucial role in the development of various organs (17). SIX1 only shows limited expression in some types of cells, including tumor cells (13, 14), implying that SIX1 might contribute to tumor lymphangiogenesis. Conversely, SIX1 has been found to play a critical role in tumor lymphangiogenesis and lymph node metastasis in many tumor types (4, 11). Nevertheless, the mechanisms underlying the modulation of VEGF-C and VEGF-D expression in the tumor milieu, as well as their effect on tumor lymphangiogenesis, have not been fully elucidated.

TGFβ is known to play an important role in tumor progression, including metastasis (12). Although the precise relationship between TGFβ and tumor lymphangiogenesis is not well known, recent studies suggest that TGFβ might exert dual effects on lymphangiogenesis. TGFβ might upregulate VEGF-C expression in some types of cells, including tumor cells (13, 14), implying that TGFβ might contribute to tumor lymphangiogenesis. Conversely, TGFβ has been found to downregulate VEGF receptor-3 (VEGFR3) in LECs and suppress LEC properties, thus inhibiting lymphangiogenesis (15, 16). So far it remains unclear whether the effects of TGFβ on LECs and VEGF-C-expressing cells can be modulated and what is the final effect of TGFβ on tumor lymphangiogenesis.

Sine oculis homeobox homolog 1 (SIX1) is a developmentally regulated homeoprotein that plays a crucial role in the development of various organs (17). SIX1 only shows limited expression in most non-neoplastic adult tissues but is frequently overexpressed in numerous malignancies (18, 19). The overexpression of SIX1 correlates with poor clinical prognosis in a variety of cancers, including breast cancer, ovarian cancer,
hepatocellular carcinoma, and rhabdomyosarcoma (20). SIX1 has been found to transcriptionally activate the expression of multiple protumorogenic genes and impair a DNA damage-induced G_{2}-M checkpoint by upregulating the expression of cyclin A1, cyclin D1, and c-Myc (21, 22). Importantly, SIX1 has been found to enhance TGF-β signaling (20) and transcriptionally activate VEGF-C expression (23), suggesting that SIX1 may have the potential to modulate tumor lymphangiogenesis.

In cervical cancer, lymphatic vessels are the major route for tumor metastasis (24). Infection by high-risk human papillomavirus (HPV), the major cause of cervical cancer (25), can significantly upregulate SIX1 expression (26). A recent study suggested that lymph node metastasis in cervical cancer correlates with higher SIX1 mRNA levels in primary tumors (27). Moreover, higher VEGF-C expression in cervical cancer is associated with higher densities of peritumoral lymphatic vessels, increased lymphatic invasion, and increased lymph node metastasis (28). Higher TGF-β1 expression is also associated with lymph node metastasis and poor prognosis of cervical cancer (29, 30). On the basis of these premises, in this study, we investigated whether SIX1 and TGF-β were responsible for modulating VEGF-C expression and lymphangiogenesis in cervical cancer. Our data demonstrated that SIX1 could enhance the activation and effect of the TGF-β-SMAD pathway in cervical cancer cells, thus increasing VEGF-C expression to promote lymphangiogenesis. Importantly, the increased production of VEGF-C not only directly promoted lymphangiogenesis but also thwarted the inhibitory effect of TGF-β on lymphangiogenesis. Therefore, tumor cells that expressed high levels of SIX1 could promote lymphangiogenesis and counteract the negative effects of TGF-β on lymphangiogenesis by increasing the expression of VEGF-C.

Materials and Methods

Cells

Human cervical squamous carcinoma cell lines Siha, C33a, and Caski were purchased from the ATCC and cultured according to their guidelines. All of these cell lines were authenticated at Shanghai Paternity Genetic Testing Center in June 2012, using short tandem repeat (STR) DNA profiling (ABI 3130xl Genetic Analyzer; Life Technologies). Human LEC (HLEC) were purchased from ScienCell and cultured in endothelial cell medium (ECM; ScienCell) with 5% FBS and endothelial growth medium supplements (VEGF-C–free). In all related experiments, the fourth passage of HLECs was used. The cells showed typical cobblestone-like morphology. More than 95% of HLECs expressed LYVE-1 and podoplanin (lymphatic markers), verified by flow cytometry.

Cell transfection

RFP/luciferase-expressing tumor cells were prepared as we described previously (31). Siha cells and C33a cells were then transfected with pcDNA3.1 control plasmid or pcDNA3.1-SIX1 expression plasmid (a kind gift from Kong-Ming Wu, Thomas Jefferson University, Philadelphia, PA) using Lipofectamine 2000 (Invitrogen) and selected with G418. shRNA lentiviral particles (GeneChem) were used to knock down the expression of SIX1, VEGF-C, and TGF-β receptor 1 (TβR1) in tumor cells. The sequences targeted by shRNAs are as follows: NshSIX1(1), 5′-CCAGCTGAGAGAGATT-3′; NshSIX1(2), 5′-CAGCGAGTCAACTAGTCA-3′; PshVEGFC(1), 5′-CCGCGACAAACA CCTTCTTTAA-3′; PshVEGFC(2), 5′-CACATTATAACAGAGAT-3′; PshTβR1(1), 5′-CTGTAATTCTGCTGTAATA-3′; PshTβR1(2), 5′-GGCTTAGTATTCTGGGAAA-3′. "N" and "P" the first letter in the names of shRNAs, indicate neomycin resistance and puromycin resistance of the vector. Nshcon and Pshcon, not targeting any known gene, were used as controls. After selection with G418 and/or puromycin, the cells with stable transfection of shRNA were used for further experiments. To downregulate the expression of SIX1, SMAD2/3, or TβR1, the corresponding siRNA (Ribobio) was transfected into tumor cells using Lipofectamine 2000 according to standard protocols.

Immunohistochemistry

Cervical squamous cell carcinoma (CSCC) samples were acquired by surgery from patients with cancer without preoperative radiotherapy or chemotherapy, which was approved by the Ethical Committee of the Medical Faculty of Tongji Medical College (Wuhan, PR China). Informed consent was obtained from all subjects. The tumor samples were used for the preparation of tissue microarray (TMA; for SIX1, VEGF-C, TGF-β1, TGF-β2, TGF-β3, and TβR1 staining) at Outo BioTech Company (Shanghai), as well as for the preparation of individual sections for LYVE-1 staining. Tissue sections were subjected to immunohistochemical (IHC) analysis as described previously (32). For semiquantitative evaluation of protein level in tissue, an immunoreactivity scoring system (HSCORE) was used (28, 31). HSCORE ≥ 2.0 was classified as a low protein level, and HSCORE > 2.0 was classified as a high protein level. Lymphatic vessel density (LVD) was determined according to the methods described by Gombos and colleagues (28) and Zhang and colleagues (33). The slides were read by two pathologists, and each data point represents the mean of the two scores. See Supplementary Methods for more details.

Western blot assay

Western blot assay was done as described previously (34). Antibodies are described in the Supplementary Methods.

In vivo lymph node metastasis

Female NOD-SCID mice (4 weeks old) were purchased from Beijing HFK Bio-Technology Co, Ltd. for studies approved by the Committee on the Ethics of Animal Experiments of Tongji Medical College. The mice were maintained in the accredited animal facility of Tongji Medical College. The mice were anesthetized by intraperitoneal injection of Nembutal (sodium pentobarbital) and were transcardially perfused with 4% paraformaldehyde. Lymph node metastasis was assessed with an optical imaging system (IVIS; Xenogen). The number of tumor cells was visualized with a fluorescent luciferase reporter transgene using IVIS SPECTRUM system (Caliper, Xenogen). To visualize lymphatic vessels, a fluorescent luciferase reporter transgene was used that expressed LYVE-1 in lymphatics.

In vivo tumor growth

Female NOD-SCID mice (4 weeks old) were purchased from Beijing HFK Bio-Technology Co, Ltd. for studies approved by the Committee on the Ethics of Animal Experiments of Tongji Medical College. The mice were maintained in the accredited animal facility of Tongji Medical College. The mice were anesthetized by intraperitoneal injection of Nembutal (sodium pentobarbital) and were transcardially perfused with 4% paraformaldehyde. Tumor growth was assessed with an optical imaging system (IVIS; Xenogen). The number of tumor cells was visualized with a fluorescent luciferase reporter transgene using IVIS SPECTRUM system (Caliper, Xenogen). To visualize lymphatic vessels, a fluorescent luciferase reporter transgene was used that expressed LYVE-1 in lymphatics.

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mouse, as suggested previously; ref. 36). The percentage of metastasis-positive lymph nodes (the ratio of positive lymph nodes to total lymph nodes) in each mouse, and the incidence of lymph node metastasis (the ratio of the mice with lymph node metastasis to total mice) in each group were calculated.

**Assay of cytokine and growth factor with antibody-based array**
Cytokines and growth factors in the culture supernatants were detected using Human Cytokine Antibody Array 9 and Human Growth Factor Antibody Array 1 (RayBiotech, Inc.) according to manufacturer’s protocol. See Supplementary Methods for more details.

**ELISA analysis**
VEGF-C in the culture supernatants of tumor cells were quantified using human VEGF-C ELISA Kit (Bender MedSystems) according to the manufacturer’s protocol.

**Assay of gene expression by real-time RT-PCR**
The relative quantity of mRNA was determined by real-time RT-PCR as described previously (31). GAPDH and EEF1A1 were chosen as reference genes, which were reported as the most reliable combination in cervical cancer (37). The primer sequences are shown in the Supplementary Methods.

**Assay of tube formation**
A total of 30 μL per well Matrigel (BD Biosciences) and 20 μL per well serum-free ECM were mixed and polymerized in 96-well plate. Then, 6 × 10^3 HLECs in 100 μL ECM were placed onto the layer of Matrigel in each well. After a 3-hour incubation, tube morphogenesis was assessed by phase contrast microscopy. Tube formation was quantified by measuring the total length of tube structures in 3 random fields. The average length of tube per field was calculated.

**Flow cytometry**
HLECs were incubated with phycoerythrin (PE)-conjugated mouse anti-human VEGFR3 antibody (R&D) or isotype control at room temperature for 0.5 hour. Flow cytometry was performed as described previously (31). The expression index was calculated using the formula: mean fluorescence × percentage of positively stained cells (31, 38).

**Other methods**
Migration assay, immunoprecipitation (IP), and chromatin immunoprecipitation (ChIP) were performed using standard protocols. See Supplementary Methods for more details.

**Statistical analysis**
SPSS (version 13.0) software package was used for statistical analysis. Results were expressed as mean value ± SEM and interpreted by one-way ANOVA. Correlation analyses were performed with Pearson correlation coefficient.
done using Spearman rank test. Difference in the ratio of lymph node metastasis was analyzed by Fisher exact probability test. Differences were considered to be statistically significant when $P < 0.05$.

Results

Lymph node metastasis of cervical cancer correlates with higher SIX1 expression at the primary site

To determine the effect of SIX1 on lymph node metastasis, we first investigated whether lymph node metastasis of cervical cancer correlated with the expression level of SIX1 in tumor. A significant increase in SIX1 expression was found at the primary site of cervical cancers with lymph node metastasis (Fig. 1A). Correspondingly, higher SIX1 expression correlated with increased peritumoral LVD in cervical cancer (Fig. 1B). The same results were obtained when SIX1 expression was quantified using real-time RT-PCR (data not shown). These results suggest that the upregulation of SIX1 in primary tumor may promote lymphangiogenesis and facilitate lymph node metastasis in cervical cancers.

SIX1 overexpression in tumor cells promotes lymph node metastasis in vivo

To demonstrate the role of SIX1 in promoting lymph node metastasis in cervical cancer, SIX1 was overexpressed in Siha cells, a CSCC cell line with low-level expression of SIX1 (Fig. 2A). The peritumoral LVD in orthotopically transplanted Siha-SIX1 tumors was significantly higher than in control Siha tumors (Fig. 2B). Because lymph node metastasis positively correlates with tumor size (35, 39), metastasis was analyzed when primary tumors reached comparable sizes, based on the dynamic monitoring of orthotopic tumor growth in living mice (Supplementary Fig. S1A). When primary tumors reached comparable sizes (Supplementary Fig. S1B), the ratio of metastasis-positive lymph nodes (Fig. 2C) and the ratio of lymph node metastasis (Fig. 2D) were both significantly increased in the Siha-SIX1 groups. The same effect was also observed in C33a cells, another CSCC cell line with low-level expression of SIX1 (Supplementary Fig. S2). Correspondingly, knockdown of SIX1 in Caski cells, a cervical cancer cell line with high levels of SIX1 expression, resulted in a decrease of peritumoral LVD and lymph node metastasis in orthotopic tumors (Fig. 2 and Supplementary Fig. S1). Taken together, these results suggest that increased expression of SIX1 in cervical cancer cells might promote lymphangiogenesis and lymph node metastasis in vivo.

SIX1 upregulates the expression of VEGF-C in CSCC cells

Because SIX1 is a nuclear protein that cannot be released from tumor cells, we next investigated whether the effect of SIX1 overexpression in tumor cells promotes lymphangiogenesis and lymph node metastasis. A, SIX1 expression in Siha or Caski cells stably transfected with the indicated vector was detected by Western blotting. Two independent cell clones of Siha-SIX1 cells were selected. Two nonoverlapping shRNAs were used to knockdown the expression of SIX1 in Caski cells. B, after orthotopic transplantation of tumors preformed by the indicated cells, lymphatic vessels ($n = 14$ per group) were visualized by staining for LYE-1. Representative images of positive staining are shown at $\times 200$ magnification (left), T, tumor. Peritumoral LVD was quantified (right). C and D, quantitative analysis of lymph node (LN) metastasis was performed as described in Materials and Methods. Lymph nodes with tumor cells (indicated by yellow arrows) were identified by tumor cell–expressed RFP when orthotopic tumors were removed. The percentage of metastasis-positive lymph node (C) and the ratio of lymph node metastasis (D) were calculated. $^*$, $P < 0.01$; $^{**}$, $P < 0.001$. 

Figure 2. Overexpression of SIX1 in cervical cancer cells promotes lymphangiogenesis and lymph node metastasis. A, SIX1 expression in Siha or Caski cells stably transfected with the indicated vector was detected by Western blotting. Two independent cell clones of Siha-SIX1 cells were selected. Two nonoverlapping shRNAs were used to knockdown the expression of SIX1 in Caski cells. B, after orthotopic transplantation of tumors preformed by the indicated cells, lymphatic vessels ($n = 14$ per group) were visualized by staining for LYE-1. Representative images of positive staining are shown at $\times 200$ magnification (left), T, tumor. Peritumoral LVD was quantified (right). C and D, quantitative analysis of lymph node (LN) metastasis was performed as described in Materials and Methods. Lymph nodes with tumor cells (indicated by yellow arrows) were identified by tumor cell–expressed RFP when orthotopic tumors were removed. The percentage of metastasis-positive lymph node (C) and the ratio of lymph node metastasis (D) were calculated. $^*$, $P < 0.01$; $^{**}$, $P < 0.001$. 

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SIX1 might be mediated by released soluble factors that promote lymphangiogenesis. To do this, cytokines and growth factors in the culture supernatants of cervical cancer cells were detected using antibody-based arrays (Supplementary Fig. S3). Other known lymphangiogenic factors absent from the arrays were analyzed by measuring mRNA levels of their corresponding genes (Supplementary Fig. S4; refs. 7, 8). The results showed that SIX1 could upregulate the expression of VEGF-C but not other factors involved in lymphangiogenesis (Fig. 3A and Supplementary Fig. S4). The expression of the VEGF-C gene was significantly increased in Siha-SIX1 cells (Fig. 3B). Knocking down SIX1 expression in Siha-SIX1 cells (Supplementary Fig. S5A) significantly reduced the expression of VEGF-C (Fig. 3B). The same effect was also observed in C33a cells (Supplementary Fig. S5) and Caski cells (Fig. 3C). Moreover, the analysis of human CSCC primary tumors indicated higher protein levels of VEGF-C in the SIX1-high group (Fig. 3D) and the positive correlation between SIX1 and VEGF-C mRNA levels (Fig. 3E). These results demonstrate that SIX1 could promote the expression of VEGF-C in cervical cancer cells.

The effect of SIX1 on lymphangiogenesis and lymph node metastasis is mediated by VEGF-C

On the basis of the above results, we next investigated the effect of tumor cells on migration and tube formation of HLECs in vitro, which are crucial for tumor lymphangiogenesis (40, 41).

Compared with corresponding control groups, migration and tube formation of HLECs were significantly promoted by the culture supernatants of SIX1-expressing tumor cells (Supplementary Fig. S6). To further clarify whether VEGF-C might mediate the effect of SIX1 on HLECs in vitro, we inhibited VEGF-C expression by expressing VEGF-C shRNA in tumor cells (Supplementary Fig. S7) or blocked VEGF-C in the culture supernatants with soluble VEGFR3. Both shRNA and VEGFR3 abolished the effects of Siha-SIX1 cells culture supernatants in promoting HLEC migration (Fig. 4A and Supplementary Fig. S8A) and tube formation (Fig. 4B and Supplementary Fig. S8A). Consistently, when VEGF-C was knocked down or blocked, knockdown of SIX1 expression in Caski cells could not further reduce the migration or tube formation of HLECs (Fig. 4A,B and Supplementary Fig. S8B). Moreover, when VEGF-C shRNA was expressed in tumors, SIX1 overexpression in Siha cells did not increase LVD, nor did SIX1 knockdown in Caski cells reduce LVD in orthotopic tumors (Fig. 4C and Supplementary Fig. S9). Similar results were obtained in C33a cells (Supplementary Fig. S10). These results indicate that the promotion of lymphangiogenesis by SIX1 is mediated by VEGF-C.

We then analyzed the clinical samples to clarify the correlation of SIX1 and VEGF-C expression with lymph node metastasis. The samples from human CSCC primary tumors were divided into four groups based on the HSCORE of SIX1 and VEGF-C staining. The rate of lymph node metastasis in tumors with high SIX1 and VEGF-C expression in the SIX1-high group (n = 25) was calculated (right). E, correlation analysis of SIX1 and VEGF-C expression in tumors. The relative expression of SIX1 and VEGF-C in 54 CSCC samples was detected by real-time RT-PCR. The expression levels of VEGF-C were plotted against the expression levels of SIX1. ***, P < 0.001; ***, P < 0.001.

Figure 3. SIX1 upregulates the expression of VEGF-C in CSCC cells. A, cytokines and growth factors in the culture supernatants from the indicated tumor cells were detected using antibody-based arrays as described in Materials and Methods. Representative results of lymphangiogenic and other factors are shown. Culture medium (DMEM with 2% FCS) was used as a blank control. See Supplementary Fig. S3 for full results and additional annotation. B and C, VEGF-C mRNA levels in Siha-SIX1 cells (B, left) or Caski cell (C, left) were detected by real-time RT-PCR. VEGF-C in culture supernatants of Siha cells (B, right) or Caski cell (C, right) was detected by ELISA. D, IHC analysis of SIX1 and VEGF-C protein in TMA of human CSCC specimens. Representative images of VEGF-C staining are shown at ×400 magnification (left); insets are the enlarged local images. The HSCORE of VEGF-C in the SIX1-low group (n = 48) and SIX1-high group (n = 29) was calculated (right). E, correlation analysis of SIX1 and VEGF-C expression in tumors. The relative expression of SIX1 and VEGF-C in 54 CSCC samples was detected by real-time RT-PCR. The expression levels of VEGF-C were plotted against the expression levels of SIX1. ***, P < 0.001; ***, P < 0.001.
metastasis in SIX1-high/VEGF-C–high patients was significantly higher than in SIX1-high/VEGF-C–low patients (Fig. 4D), suggesting that VEGF-C was required for SIX1 to promote lymph node metastasis. Consistent with this, when VEGF-C was knocked down in cervical cancer cells, lymph node metastasis of orthotopic tumors was not affected by either increasing SIX1 expression in Siha or C33a cells (Fig. 4E and Supplementary Fig. S11A) or decreasing SIX1 expression in Caski cells (Fig. 4E and Supplementary Fig. S11B). Taken together, these results demonstrate that the promotion of lymph node metastasis by SIX1 is mediated by VEGF-C.

SIX1 coordinates with TGFβ to promote VEGF-C expression

A recent study showed that SIX1 could bind to the VEGF-C gene promoter and directly activate transcription of VEGF-C (23). Interestingly, the relative levels of VEGF-C mRNA in SIX1-high clinical specimens were much higher than those in cell lines as shown in Fig. 3B, C, and E. To further confirm the differences between tumors in vivo and tumor cells cultured in vitro, we compared the expression of VEGF-C in cell lines with that in orthotopically transplanted tumors. Although SIX1 mRNA levels in tumors were similar to corresponding cells cultured in vitro (Supplementary Fig. S12A), VEGF-C mRNA levels in SIX1-expressing tumors were indeed significantly higher than those in SIX1-expressing cells cultured in vitro. However, VEGF-C mRNA levels in control Siha tumors and Caski-NshSIX1 tumors were similar to those in corresponding cells cultured in vitro (Fig. 5A and Supplementary Fig. S12B). In addition, low levels of VEGF-C were observed in some of the SIX1-high clinical samples as shown in Fig. 4D. These results suggest that other factor(s) in the tumor milieu might be necessary for SIX1 to induce a high level of VEGF-C expression.

It has been shown that TGFβ1 can induce VEGF-C expression in some cell types (13) and that SIX1 can enhance TGFβ signaling (20). This raises the question whether SIX1 coordinates with TGFβ to increase VEGF-C expression. To answer this question, we first analyzed the prevalence of TGFβ members in CSCC specimens (Supplementary Fig. S13). The results showed that the protein levels of both TGFβ1a and TGFβ2 were lower in the SIX1-high/VEGF-C-low groups, whereas the protein level of TGFβ3 was low across all conditions (Fig. 5B). Because each isoform of TGFβ is capable of mediating TGFβ signaling and exerts overlapping biologic functions, the expression of TGFβ was considered to be high if at least one member of the family was highly expressed in clinical samples (42, 43). Interestingly, higher expression of TGFβ correlated with higher expression of VEGF-C in SIX1-high clinical samples, but not in SIX1-low samples (Fig. 5C). Consistent with this result, TGFβ1 (Fig. 5D...
SIX1 Coordinates with TGFβ1 in Tumor Lymphangiogenesis

Figure 5. SIX1 coordinates with TGFβ1 to promote VEGF-C expression. A, VEGF-C mRNA levels from the indicated cells cultured in vitro and the corresponding orthotopic tumors formed in vivo were detected by real-time RT-PCR analysis. B and C, after IHC analysis of SIX1, VEGF-C, and TGFβ1 in TMA of human CSCC specimens, the levels of TGFβ1 and VEGF-C were further analyzed. The specimens with a high level of SIX1 were divided into VEGF-C-low and VEGF-C-high groups. The HSCORE of each type of TGFβ1 in the SIX1-high/VEGFC-low group (n = 10) and SIX1-high/VEGFC-high group (n = 15) was calculated (B). Within the SIX1-high and SIX1-low groups, the samples were further subgrouped into TGFβ1-low and TGFβ1-high. The expression of TGFβ1 was considered to be high if at least one member of TGFβ1 was highly expressed (HSCORE > 2.0). The TGFβ1-low groups consisted of samples in which low levels of all three TGFβ1 members were expressed. The HSCORE of VEGF-C in each group was calculated (C). D, the expression of the VEGF-C gene in the indicated cells was detected by real-time RT-PCR after treatment with TGFβ1 at different concentrations for 24 hours. E, VEGF-C mRNA levels in the indicated cells cultured in vitro and the corresponding orthotopic tumors formed in vivo were detected by real-time RT-PCR. F and G, after orthotopic transplantation of tumors preformed by the indicated cells (n = 14 per group), peritumoral LVD (F) and the ratio of lymph node (LN) metastasis (G) were calculated as described in Materials and Methods. n.s., not significant. **, P < 0.01; ***, P < 0.001.

and Supplementary Fig. S14A) and TGFβ2 (data not shown) negligibly increased VEGF-C expression in the cervical cancer cell lines with low levels of SIX1 expression but induced much higher expression of VEGF-C in SIX1-expressing cell lines in a dose-dependent manner. Moreover, when tumor cells were pretreated with TGFβ1, the promotion of tube formation of HLECs by supernatants from SIX1-expressing cells was significantly augmented, which was dependent on VEGF-C (Supplementary Fig. S14B). These results indicate that SIX1 can enhance the response of tumor cells to TGFβ1, thus promoting VEGF-C expression.

To determine the role of TGFβ1 signaling in SIX1-induced VEGF-C expression and lymphangiogenesis in vivo, we used TβRI shRNAs to inhibit TGFβ1 signaling in tumor cells (Supplementary Fig. S15A). When TβRI was knocked down in SIX1-expressing cells, the differences in VEGF-C expression between tumors in vivo and tumor cells cultured in vitro were abolished (Fig. 5E and Supplementary Fig. S15B), indicating that TGFβ1 signaling was responsible for further promoting VEGF-C expression in vivo. Consistently, the promoting effect of SIX1 on lymphangiogenesis (Fig. 5F and Supplementary Fig. S15A) and lymph node metastasis (Fig. 5G and Supplementary Fig. S15B) was significantly inhibited when TβRI was knocked down. These results demonstrate that TGFβ1 signaling is necessary for SIX1 to both induce higher expression of VEGF-C in tumor cells and promote lymphangiogenesis and lymph node metastasis of cervical cancer cells in vivo.

SIX1 enhances the activation and effect of the SMAD pathway to promote VEGF-C expression

TGFβ1 induced much higher expression of VEGF-C in tumor cells with higher expression of SIX1 than in those with lower expression of SIX1. Therefore, we further investigated whether SIX1 might modulate the effects of TGFβ1. Both SMAD2 and SMAD3 could bind to the VEGF-C gene in the indicated cells was detected by real-time RT-PCR after treatment with TGFβ1 at different concentrations for 24 hours. E, VEGF-C mRNA levels in the indicated cells cultured in vitro and the corresponding orthotopic tumors formed in vivo were detected by real-time RT-PCR. F and G, after orthotopic transplantation of tumors preformed by the indicated cells (n = 14 per group), peritumoral LVD (F) and the ratio of lymph node (LN) metastasis (G) were calculated as described in Materials and Methods. n.s., not significant. **, P < 0.01; ***, P < 0.001.

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TGFβ-SMAD2, SMAD3, and p-SMAD3 were detected by Western blotting. C, ChIP-qPCR assay. ChIPs were performed using the indicated cells treated with siRNA. After 36 hours, the cells were stimulated with TGFβ1 (1 ng/mL) for 1 hour. Real-time PCR was then performed to detect a series of regions upstream of the transcriptional start site of SIX1. D, Siha control cells, Siha-SIX1 cells, Caski control cells, and Caski-NshSIX1 cells were stimulated with TGFβ1 (1 ng/mL) for 1 hour. p-SMAD2, p-SMAD3, and p-SMAD3 were detected by Western blotting using human anti-SMAD2 or human anti-SMAD3 antibodies (left) or immunoprecipitated with human anti-SMAD2 or human anti-SMAD3 antibodies and analyzed by Western blotting using an anti-SIX1 antibody (right). ***, P < 0.001.

SIX1 could also bind to the VEGF-C gene promoter (Supplementary Fig. S19). Knocking down the expression of SMAD2/3 did not reduce the binding of SIX1 to the VEGF-C promoter (Supplementary Fig. S20). Intriguingly, co-IP assays showed that SIX1 protein could interact with SMAD2/3 (Fig. 6F), suggesting that the interaction of SIX1 and SMAD2/3 might also be required for inducing higher expression of VEGF-C. Taken together, these results suggest that SIX1 can enhance TGFβ-induced activation of SMAD2/3 and might cooperate with the SMAD pathway to promote VEGF-C expression.

SIX1 modulates the effect of TGFβ on lymphangiogenesis

The above data indicated that TGFβ could stimulate SIX1-expressing tumor cells to increase the production of VEGF-C. However, TGFβ has a negative effect on lymphangiogenesis due to its inhibitory effect on the expression of VEGFR3, the receptor of VEGF-C, in HLECs (15). This led to the question whether VEGF-C could promote tube formation of HLECs in the presence of TGFβ. Indeed, TGFβ1 could attenuate the promotion of HLEC tube formation by VEGF-C (Fig. 7A). Nevertheless, VEGF-C could attenuate the inhibitory effect of TGFβ1 on VEGFR3 expression in a dose-dependent manner (Fig. 7B), which is consistent with a previous finding that VEGF-C not only binds VEGFR3 but also stimulates VEGFR3 expression (44, 45). Importantly, at higher concentrations, VEGF-C could still promote tube formation in presence of TGFβ1 (Fig. 7C) or TGFβ2 (data not shown). These results suggest that higher concentrations of VEGF-C can antagonize the inhibitory effect of TGFβ on HLECs.

To clarify whether the increased VEGF-C in SIX1-expressing tumors could modulate the final effect of TGFβ on lymphangiogenesis, we used the TβRI inhibitor LY364947 to block TGFβ signaling in vivo. LY364947 negligibly affected VEGF-C expression in Siha-3.1 and Caski-NshSIX1 tumors (Fig. 7D), but could increase LVD in these same tumors (Fig. 7E), suggesting that TGFβ might inhibit lymphangiogenesis through its negative effect on LECs. However, LY364947 treatment significantly reduced the expression of VEGF-C gene in SIX1-expressing tumors (Fig. 7D) and also reduced LVD (Fig. 7E) and lymph node metastasis (Fig. 7F). These results demonstrated that the final effect of TGFβ on lymphangiogenesis of SIX1-expressing tumor was to promote this process. Moreover, when VEGF-C was knocked down in...
SIX1-expressing orthotopic tumors (Fig. 7D), intraperitoneal injection of LY364947 increased LVD (Fig. 7E). These results indicate that different levels of VEGF-C in tumor can result in different effects of TGFβ on lymphangiogenesis.

**Discussion**

Lymphatic dissemination of tumor cells has been known to correlate with peritumoral LVD (2, 40). The data in this study show that the increased expression of SIX1 in tumor cells can increase peritumoral LVD and promote lymph node metastasis in cervical cancers. We found that SIX1 altered the effect of TGFβ on tumor lymphangiogenesis by coordinating with TGFβ to promote the expression of VEGF-C in tumor cells. The increased production of VEGF-C by tumor cells not only promoted the migration and tube formation of LECs but also thwarted the inhibitory effect of TGFβ on lymphangiogenesis. Therefore, the increase of SIX1 expression in tumor cells enhanced the promotion of lymphangiogenesis by TGFβ while attenuating its inhibitory effects, thus promoting lymph node metastasis of tumor cells.

VEGF-C and VEGF-D are important growth factors for promoting lymphangiogenesis in various contexts, although most of their effects are deemed to be secondary to the induction of VEGF-C and VEGF-D (8, 47). Nonetheless, the data in this study show that VEGF-C is the primary growth factor mediating the promotional effect of SIX1 on tumor lymphangiogenesis. SIX1 could upregulate the expression of VEGF-C but not other lymphangiogenic factors. VEGF-C mediated the promotion of migration and tube formation of HLECs by SIX1-expressing tumor cells in vitro. Either inhibiting VEGF-C expression or blocking the function of VEGF-C could abolish the effect of SIX1. VEGF-C also mediated the promotion of lymphangiogenesis by SIX1 in vivo. Moreover, VEGF-C expression was required for SIX1 to promote lymph node metastasis as shown by the analyses using human clinical samples and a mouse lymphatic metastasis model. Therefore, upregulating VEGF-C expression is an important mechanism by which SIX1 promotes lymphangiogenesis and lymph node metastasis in cervical carcinoma.

In this study, we show that SIX1 alone can slightly promote the expression of VEGF-C in tumor cells. This is consistent with a previous report demonstrating that SIX1 can bind to the VEGF-C promoter and transcriptionally activate the VEGF-C gene in breast cancer cells (23). The regulatory effect of SIX1 alone on VEGF-C expression was limited. Intriguingly, both TGFβ signaling and the overexpression of SIX1 were required for inducing significantly higher expression of VEGF-C in...
tumor cells. Therefore, the coordination of SIX1 and TGFβ is crucial for the high expression of VEGF-C in tumors.

Although TGFβ has the potential to modulate VEGF-C expression (48), its effect on VEGF-C expression in tumor cells remains uncertain. TGFβ1 has been found to slightly increase VEGF-C expression in the pancreatic adenocarcinoma cell line PANC-1 (14), whereas no significant correlation has yet been found between the mRNA levels of TGFβ1 and VEGF-C in adenocarcinoma of the small intestine (49). The data in this study suggest that the differential effects of TGFβ on VEGF-C expression in different tumor cells might be due to the different expression levels of SIX1 in those cells. Our data in this study show that SIX1 can coordinate with TGFβ to induce higher expression of VEGF-C through two pathways. First, SIX1 can enhance TGFβ signaling by upregulating TβR1 expression, which promotes the activation of SMAD2/3 and its binding to the VEGF-C promoter, thus inducing higher expression of VEGF-C. Second, SIX1 could bind to the VEGF-C promoter and interact with SMAD2/3, suggesting that SIX1 might also cooperate with SMAD2/3 to modulate VEGF-C expression. In tumor cells with lower expression of SIX1, the expression of VEGF-C was inconsiderably influenced by TGFβ stimulation, although SMAD2/3 could be phosphorylated and bind to the VEGF-C promoter. Therefore, SIX1 is necessary for TGFβ to induce higher expression of the VEGF-C gene.

Although TGFβ might promote lymphangiogenesis by promoting VEGF-C expression, it also has the potential to inhibit lymphangiogenesis by inhibiting VEGFR3 expression in LECs (15). However, the data here revealed that the inhibitory effect of TGFβ1 on LECs could be attenuated if the VEGF-C concentration was increased. VEGF-C not only binds VEGFR3 to directly promote lymphangiogenesis but also upregulates VEGFR3 expression as shown by our data and others (44, 45). The stimulation of HLECs with TGFβ1 alone significantly inhibited tube formation in vitro, whereas simply increasing the concentration of VEGF-C attenuated the inhibitory effect of TGFβ1. Together, these results suggest that the excess production of VEGF-C is sufficient to antagonize the negative effect of TGFβ on lymphangiogenesis. Therefore, the effect of TGFβ on lymphangiogenesis might be negative, whereas over-expression of SIX1 in tumor cells might counteract the negative effects of TGFβ on lymphangiogenesis in an indirect manner via VEGF-C.

In summary, this study demonstrated that SIX1 can coordinate with TGFβ to promote tumor lymphangiogenesis by promoting VEGF-C expression in tumor cells. Considering the ubiquitous and complex functions of TGFβ and its regulatory diversity in tumors (50), overexpression of SIX1 might be one of the mechanisms that influence the biologic function of TGFβ. The data here suggest that blocking TGFβ signaling may have significant therapeutic value for preventing the metastasis of SIX1-positive CSCC. In the case of SIX1-negative patients, SIX1 overexpression in the cell clones might arise during the process of tumor development. Therefore, blocking TGFβ signaling can also be considered as a potential component of a comprehensive strategy for tumor therapy.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): W. Wang

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