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Original Article

SOX1 inhibits lung cancer angiogenesis by direct regulation of IGFBP1/MIF pathways

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Abstract: Previous studies have demonstrated that SOX1 silencing enhances cisplatin-mediated autophagy in NSCLC and restoration of SOX1 inhibits cell migration by regulating actin cytoskeletal remodeling in NSCLC. However, the function and potential mechanisms of SOX1 in angiogenesis have not yet been investigated. This study found that ectopic expression of SOX1 could inhibit angiogenesis, in both *in vitro* and *in vivo* conditions. Global mRNA expression was then studied in A-1 cells, with or without enforced expression of SOX1, using a microarray approach. SOX1 inhibited a set of genes associated with cancer angiogenesis, with IFGBP1 the most reduced gene. This study found that SOX1 directly interacted with, and repressed, the IGFBP1 promoter. Moreover, analysis of primary NSCLC samples revealed an inverse correlation between SOX1 and IGFBP1 levels. Finally, it was demonstrated that direct regulation of IGFBP1 was critical for SOX1-mediated angiogenesis inhibition in NSCLC. This study also found that MIF was the essential tumor-derived soluble factor in mediating IGFBP1-induced angiogenesis. Secreted IGFBP1 not only promotes endothelial tube formation, in a paracrine manner, but also acts as a autocrine growth factor to increase expression of MIF. Taken together, these findings suggest that overexpression of SOX1 inhibits angiogenesis by direct regulation of IGFBP1/MIF pathways in NSCLC.

Keywords: Non-small cell lung cancer, SOX1, IGFBP1, MIF, angiogenesis

Introduction

Growing evidence has suggested that active angiogenesis, a critical step in cancer development and progression, is responsible for rapid recurrence and poor prognosis in patients with non-small cell lung cancer (NSCLC) [1]. Tumor angiogenesis facilitates cancer cells to easily access blood circulation by providing adequate blood vessel supply [2]. Inhibition of tumor angiogenesis represents an attractive therapeutic approach for treatment of malignancies [3]. For example, antiangiogenic therapy with bevacizumab, which targets VEGF, has been shown to improve survival in patients with lung cancer [4]. In the complex regulation of angiogenesis, many pro-angiogenic factors are involved. For example, macrophage migratory inhibitory factor (MIF), a proinflammatory cytokine, has been reported to participate in carcinogenesis, disease prognosis, tumor cell proliferation, invasion, and tumor-induced angiogenesis in many cancers [5]. MIF has been shown to promote ovarian cancer progression via increasing angiogenesis [6]. Insulin-like growth factor-binding protein 1 (IGFBP1) plays critical roles not only in development, metabolism, and homeostasis, but also in cell cycle progression, differentiation, apoptosis, and growth in normal and cancer cells [7]. IGFBP1 has been identified as a novel mediator of angiogenesis. Silencing IGFBP1 expression in microglial cells, or its neutralization by an antibody, reduces the ability of supernatants, derived from microglial cells treated with glioma cell-conditioned medium, to induce angiogenesis [8].

Many studies have revealed that SOX1 plays a crucial role in various physiological processes, in normal and malignant tissues, including cell development, proliferation, apoptosis, migration, and invasion [9]. SOX1 encodes a transcription factor implicated in the regulation of

embryonic development and determination of cell fate [10]. SOX1 has been identified as a tumor suppressor gene in hepatocellular cancer [11]. Previous studies have demonstrated that SOX1 silencing enhances cisplatin-mediated autophagy in NSCLC [12] and restoration of SOX1 inhibits cell migration by regulating actin cytoskeletal remodeling in NSCLC [13]. However, the function and potential mechanisms of SOX1 in angiogenesis have not yet been investigated.

This present study demonstrates the important role of SOX1 overexpression in inhibiting tumor angiogenesis. IGFBP1, acting as a direct target of SOX1, antagonized effects induced by SOX1 overexpression in NSCLC cells. Secreted IGFBP1 not only promotes endothelial tube formation, in a paracrine manner, but also acts as an autocrine growth factor to increase expression of MIF. IGFBP1-induced MIF contributes to IGFBP1-induced angiogenesis in NSCLC.

Material and methods

Patient samples and cell lines

Tumor samples were collected from the biopsy specimens of 50 patients with lung cancer, in the Department of Respiratory Medicine, the First Affiliated Hospital of Henan University of Traditional Chinese Medicine, China. Non-tumor samples from the macroscopic tumor margin were isolated, at the same time, and used as matched adjacent non-neoplastic tissues (> 3 cm). All samples were obtained with informed consent and procedures were approved by the Institutional Review Board of the hospital. The two different established human lung cancer cell lines used in the study (A-1 and A549) were maintained in DMEM with 10% FBS and 1% streptomycin/penicillin antibiotics.

In vivo tumor angiogenesis assay

For *in vivo* tumor formation and angiogenesis assay, a xenograft model of NSCLC was established using A549 cells. Five weeks later, tumors were removed, fixed in formalin, and embedded in paraffin. Microvessel density (MVD) in tumor tissues was evaluated based on immunostaining by CD34. Microvessels stained by CD34 were assessed by light microscopy in areas of tumor sections containing the highest vascular density. The highly vascular areas were identified by scanning tumor sections at a low power field (× 40 and then × 100 magnification). After the five areas of highest neovascularization were identified, CD34-positive vessels were manually counted at × 200 magnification.

Gene expression profiling

To identify differentially expressed genes in A-1/SOX1 and A-1/Vector cells, total RNA was amplified, labeled, and purified using GeneChip 3'IVT Express Kit (Cat#901229, Affymetrix), following manufacturer instructions to obtain biotin labeled cRNA. Array hybridization and washings were performed using GeneChip® Hybridization, Wash, and Stain Kit (Cat#900-720, Affymetrix) in Hybridization Oven 645 (Cat#00-0331-220 V, Affymetrix) and Fluidics Station 450 (Cat#00-0079, Affymetrix), according to manufacturer instructions. Raw data were normalized by MAS 5.0 algorithm. Significant differentially expressed genes were identified between the two groups using criteria of P value < 0.05 and $\log_2$ fold change (FC) > 0.5 with the R package (version 1.0.2, R Core Team, Vienna, Austria).

Expression levels of mRNA in A-1/SOX1 and A-1/Vector cells were detected by qRT-PCR analysis, according to previous descriptions.

Luciferase reporter assay

Direct targeting of the promoter of IGFBP1 by SOX1 was tested by dual luciferase assay. The experiment was performed by cloning a promoter region of IGFBP1 upstream of a firefly luciferase gene and comparing expression of the luciferase in the presence and absence of SOX1 expression vectors in HEK293T cell lines. Initially, the promoter fragment of IGFBP1 containing the predicted SOX1 binding site was amplified by PCR. Primer pairs used for its amplification created XhoI and ECORV restriction enzyme recognition sites at 5' and 3' ends, respectively. The fragment was cloned upstream of the firefly luciferase reporter gene in pGL3 vector (Promega). The cloned fragment was sequenced for verification of accuracy. Cultured cells were co-transfected with pGL3-IGFBP1 recombinant plasmid, pRL-TK (Promega Corporation), and empty pCMV-SOX1 vector. PRL-TK contained the renilla luciferase gene adjacent to the thymidine kinase promoter and pCMV-SOX1 expressed SOX1 under the constitutively expressed cytomegalovirus pro-

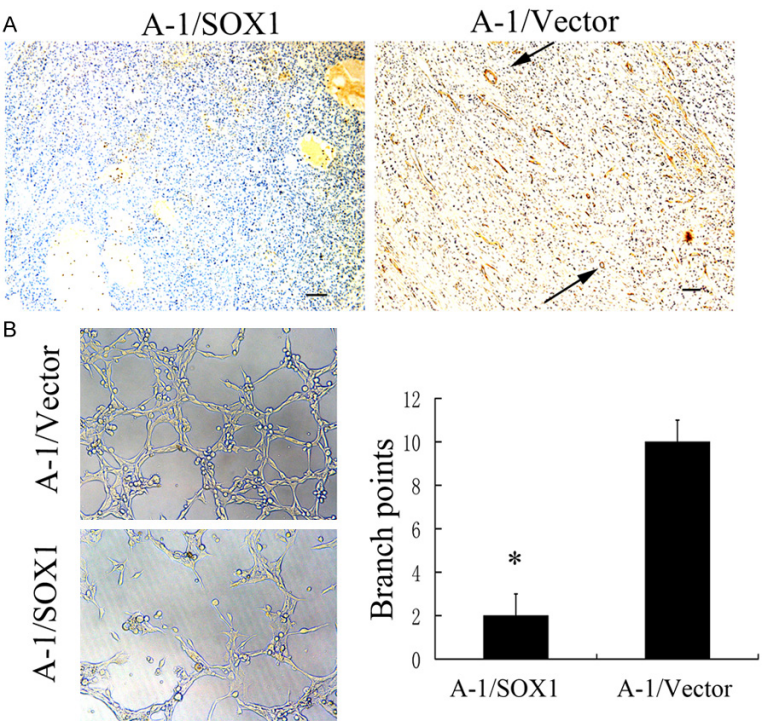

Figure 1. SOX1 inhibits angiogenesis in NSCLC. A. Evaluation of angiogenesis in tumors consisting of A-1/SOX1 or A-1/vector cells. Microvessel density (MVD) was determined by quantifying CD34-positive microvessels in tumor sections. Scale bar = 100 μm. Magnification is × 100 by light microscopy. B. Capillary tube formation assays of HUVECs with the culture medium of A-1/SOX1 and A-1/Vector cells. The number of the intersections between branches of assembled endothelial cell networks was observed at × 100 magnification. Branch points numbers were calculated (*$p < 0.05$).

moter. Transfections were performed in 24-well plates. Additional control transfections included recombinant pGL3-IGFBP1 + pRL-TK and mock transfections without vectors. All transfections were done in triplicate in HEK293T cells. Transfection reactions were performed using 500 ng of plasmid DNAs, Lipofectamine LTX reagent (Invitrogen), and 2 × 10^5 cells. Forty-eight hours after transfection, Firefly and Renilla luciferase activity levels were measured using dual luciferase assay (Promega Corporation), according to manufacturer instructions.

Antibody array detection

In total, 1 × 10^6 A-1 cells were plated into 10-cm culture dishes for 24 hours. The medium was replaced with serum deprived media containing 0.1% fetal bovine serum and cells were cultured for 48 hours. Culture supernatants were collected, centrifuged at 1000 × g, and dialyzed with 2 l of 1 × phosphate-buffered saline (pH 8) twice overnight at 4°C. Samples were labeled with biotin and incubated with Human L1000 Antibody Arrays (R&D System, #ARY005B). Representative images from the two independent experiments were shown.

Tube formation

HUVECs (2 × 10^4) were plated onto Matrigel-coated (10 mg/ml) 12-well plates with condition media of A549 cells. After 12 hours of incubation at 37°C, HUVECs were fixed with 4% paraformaldehyde and formation of capillary-like structures was captured under a light microscope. The number of branch points of the tube structures, representing the degree of angiogenesis, was counted in three fields at 100 × magnification.

In vivo metastatic model

Nude mice (4 to 5 weeks old) were maintained in SPF Laboratory Animal Central. All animal maintenance and procedures were carried out in accordance with the Institute's guidelines regarding animal experimentation. A549/IGFBP1 and A549/Vector cells (1 × 10^6) were injected into tail veins of mice. Eight weeks later, the mice were killed, and lung tissues were fixed, paraffin embedded, and 5 μm tissue sections were stained with hematoxylin and eosin (H&E). The number of macroscopic and microscopic metastatic nodules in the lungs was counted.

Statistical analysis.

All statistical analyses were carried out with SPSS Statistics 23.0 and *P*-values < 0.05 were considered statistically significant.

Results

Ectopic expression of SOX1 inhibits angiogenesis in both in vitro and in vivo conditions in NSCLC

A previous study demonstrated that all nude mice injected with A-1/SOX1 and A-1/vector

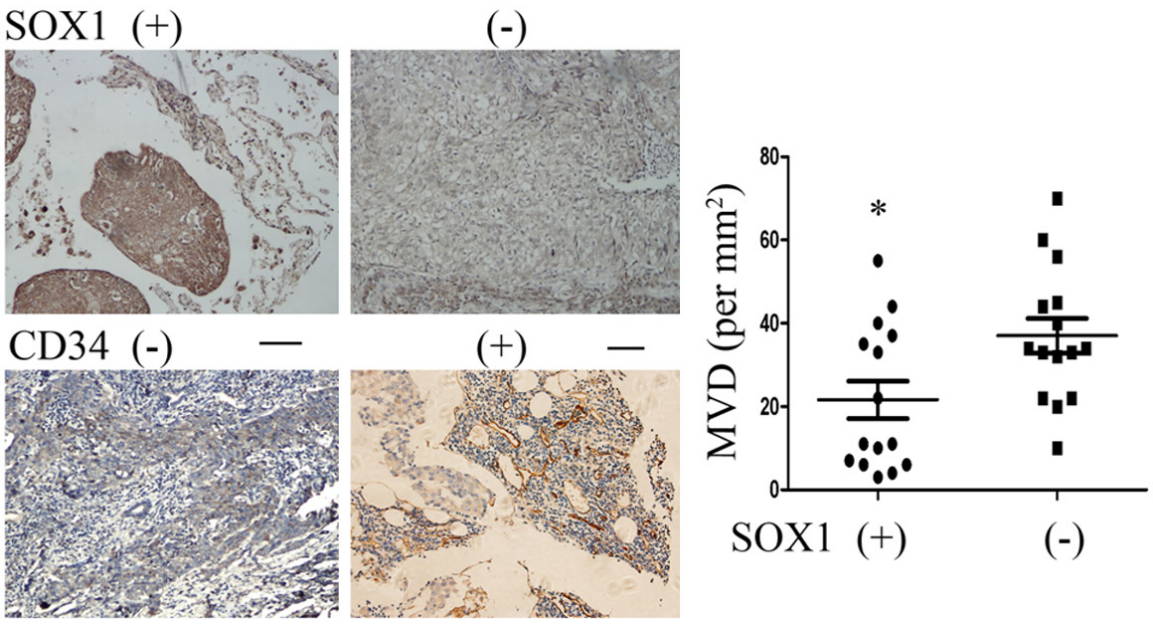

Figure 2. Representative immunohistochemical staining of SOX1 and CD34 in NSCLC. Correlation of SOX1 levels with MVD was determined by quantifying CD34-positive microvessels in NSCLC sections. Scale bar = 100 μm. Magnification, × 100 by light microscopy.

Table 1. SOX1 downregulated genes in A-1 cells identified by microarray

Symbol	Gene name	Fold change
WISP2	WNT1 inducible signaling pathway protein 2	0.532
CARS	Cysteinyl-tRNA synthetase	0.432
TM4SF1	Transmembrane 4 L six family member 1	0.478
IGFBP1	Insulin like growth factor binding protein 1	0.09
STC2	Stanniocalcin 2	0.545
CLDN1	Claudin 1	0.765

cells developed palpable tumors. There were no significant differences between A-1/SOX1 and A-1/Vector cells in cell proliferation rate, as A-1/SOX1 cells showed no growth advantage over A-1/Vector cells. However, it was found that ectopic expression of SOX1 inhibited tumor blood vessel formation, as indicated by reduced CD34 staining compared with tumors established by A-1/Vector cells (**Figure 1A**). These results suggest that overexpression of SOX1 in NSCLC inhibits tumor cell angiogenesis. To further determine the role of SOX1 in NSCLC tumor cell angiogenesis, condition medium of A-1/SOX1 and A-1/Vector cells was collected and tube formation assays were performed. As a result, the culture medium of A-1/SOX1 cells significantly inhibited capillary tube formation of HUVECs (**Figure 1B**).

Clinical evidence of SOX1 involvement in tumor angiogenesis of NSCLC

To evaluate the involvement of SOX1 in tumor angiogenesis of NSCLC, clinically, immunohistochemical staining for CD34 was used in 30 human NSCLC tissue samples, calculating the number of CD34-positive microvessels in the tumor area. Correlation between SOX1 levels and MVD was analyzed. Immunohistochemical staining showed that mean MVD was inversely associated with SOX1 levels (**Figure 2**). These results imply that SOX1 was involved in tumor angiogenesis of NSCLC.

Identification of SOX1 target genes in NSCLC cells

To investigate mechanisms by which SOX1 overexpression inhibits angiogenesis, A-1/SOX1 and A-1/Vector cells were compared using Affymetrix gene chips. In total, 171 different genes were significantly upregulated (> 2-fold increase, $p < 0.05$) and 86 were significantly downregulated (< -2 fold decrease, $p < 0.05$). A survey of the current literature indicated that many of the downregulated genes

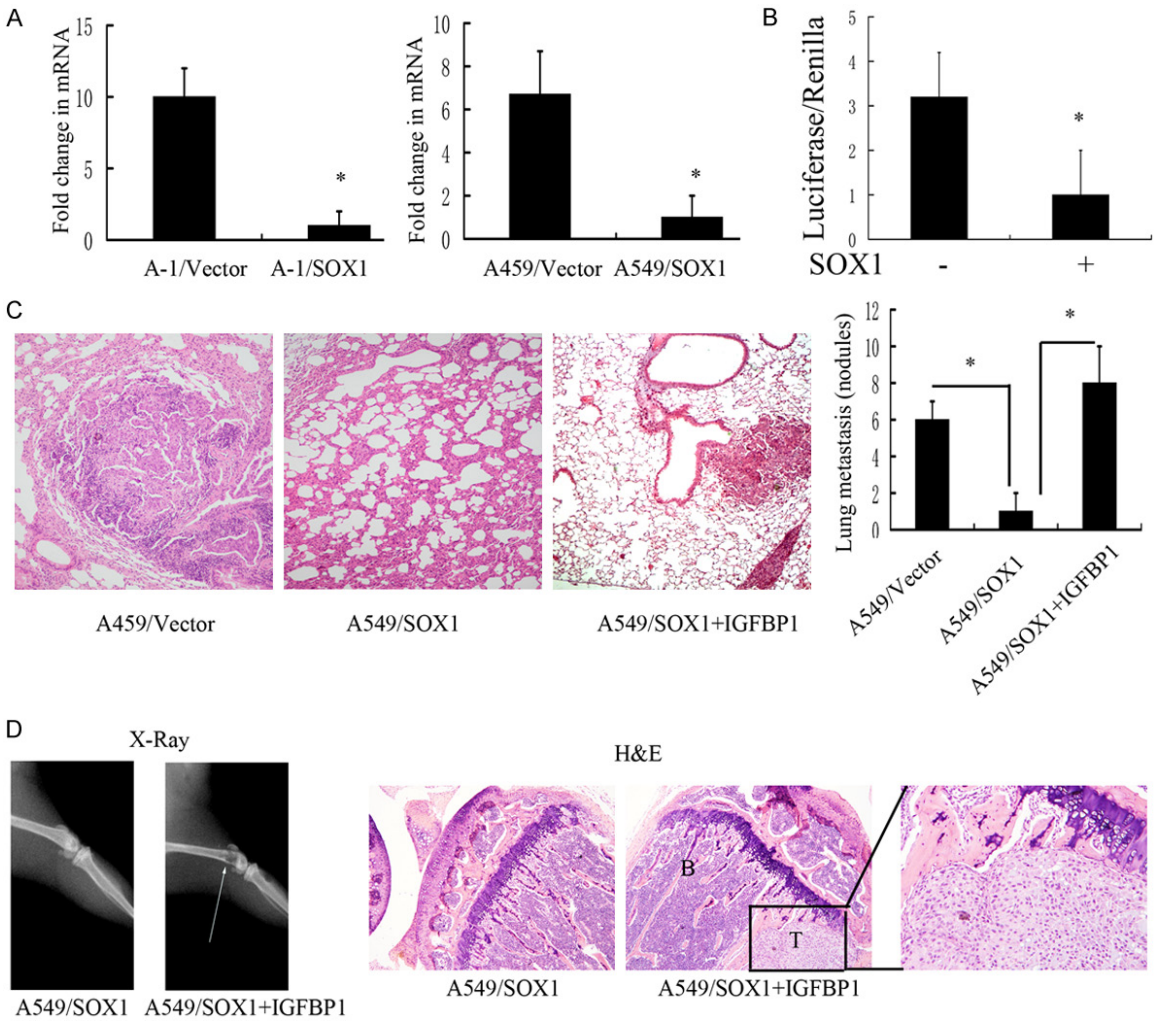

Figure 3. SOX1 directly represses the transcription of IGFBP1. A. IGFBP1 mRNA levels were examined after SOX1 overexpression in A-1 and A549. B. Luciferase assay was carried out to assess the effects of increasing amount of SOX1 on the luciferase activity of pGL3-IGFBP1 plasmid in 293T cells (*$p < 0.05$). C. IGFBP1 elevates metastatic potential of NSCLC cells *in vivo* by counteracting the function of SOX1. Representative gross view of the lungs with metastatic nodules of different groups (left). The amount of the pulmonary metastatic lesions was counted in individual group (right). D. X-ray and IHC images of bone lesions invaded by NSCLC cells from a representative mouse in each group. Arrows indicate osteolytic bone lesions in x-ray.

have been linked to cancer (**Table 1**). Compared with A-1/Vector cells, genes for WNT1 inducible signaling pathway protein 2 (WISP2), cysteinyl-tRNA synthetase (CARS), transmembrane 4 L six family member 1 (TM4SF1), insulin like growth factor binding protein 1 (IGFBP1), stanniocalcin 2 (STC2), and claudin 1 (CLDN1) were downregulated in A-1/SOX1 cells. Based on biological function and involvement in cancer angiogenesis, three proteins having the highest scores (TM4SF1, IGFBP1, and STC2) were selected for further investigation [14-16]. The results of this microarray were validated by measuring mRNA levels of selected genes using qRT-PCR to confirm that SOX1 decreases expression of selected genes. Respective mRNA expression of TM4SF1, IGFBP1, and STC2 in A-1/Vector cells was 0.47, 0.10, and 0.78 times that in A-1/SOX1 cells.

SOX1 activates IGFBP1 promoter and decreases IGFBP1 transcription

The most highly downregulated gene, IGFBP1 (insulin like growth factor binding protein-1), was decreased-10-fold by SOX1 in the microarray. qRT-PCR confirmed that SOX1 decreased IGFBP1 mRNA in A-1 and A549 cells (**Figure 3A**). To further confirm that downregulation of IGFBP1 by SOX1 occurred at the transcription level, this study cloned the 2.0-kb region upstream of IGFBP1 gene into the luciferase

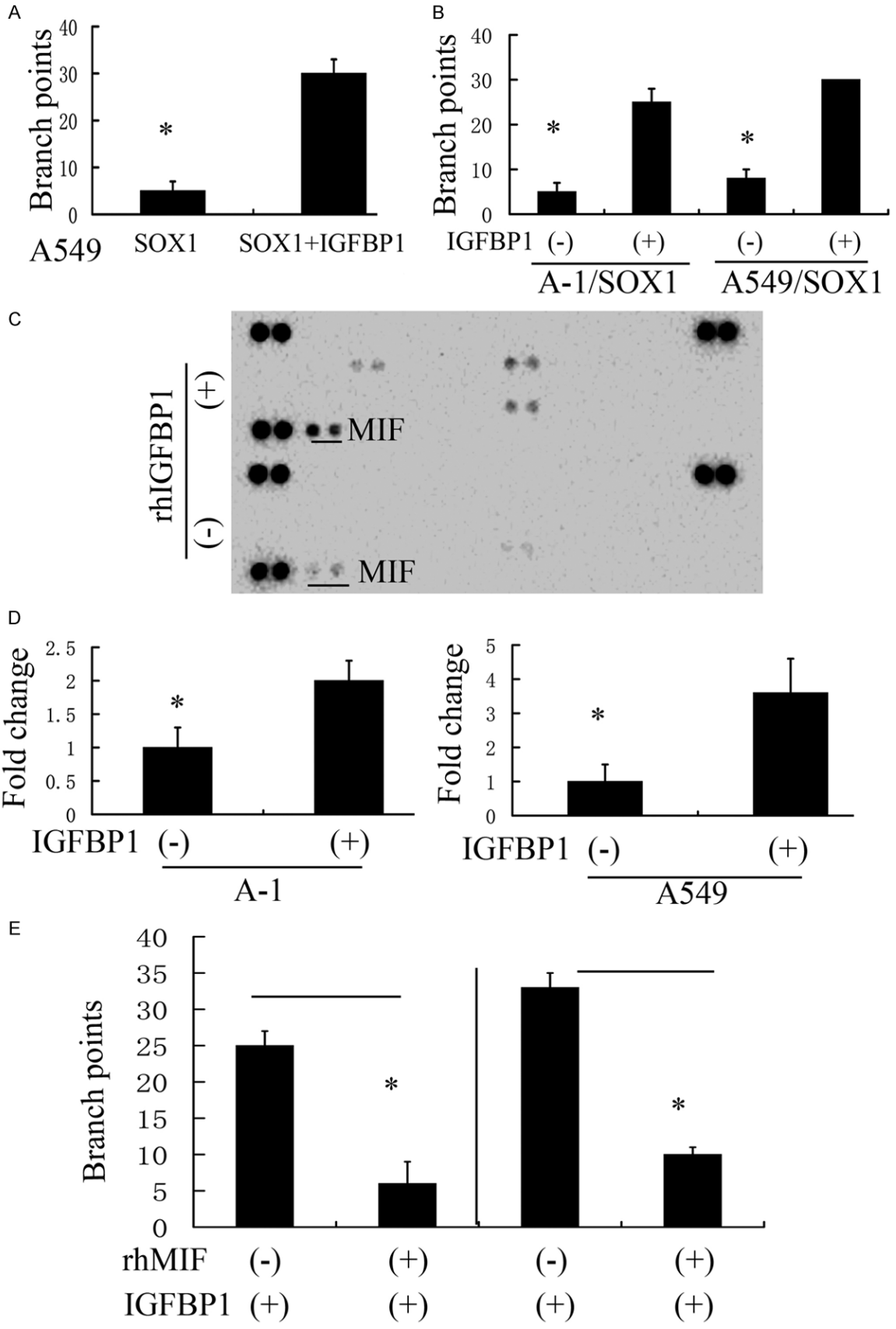
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Figure 4. SOX1 inhibits angiogenesis via IGFBP1/MIF pathways in NSCLC. A. Capillary tube formation assays of HUVECs with the culture medium of the A549/SOX1 and A549/SOX1 + IGFBP1 cells. IGFBP1 overexpression counteracts SOX1-inhibited angiogenesis in A549 cells (*$p < 0.05$). B. rhIGFBP1 simulation antagonized the effects on the abilities of capillary tube formation of HUVECs induced by SOX1 upregulation in A-1/SOX1 and A549/SOX1 cells (*$p < 0.05$). C. Cells were left untreated or treated with rhIGFBP1 for 12 hours and lysates applied to antibody array. Upregulated proteins were highlighted. Representative images from the two independent experiments are shown. D. Protein levels of MIF in the supernatant of A-1 and A549 cells were measured by ELISA. All results are from three independent experiments (*$p < 0.05$). E. MIF antibody antagonized the effects on the abilities of capillary tube formation of HUVECs induced by IGFBP1 simulation in A-1 and A549 cells (*$p < 0.05$).

reporter vector pGL3, testing the effects of SOX1 on this promoter's activity by luciferase assay. As shown in **Figure 3B**, SOX1 substantially repressed the promoter activity of IGFBP1 gene. The function of IGFBP1 in lung cancer remains largely unknown. To further test combined biologic effects of SOX1 and IGFBP1, A549/SOX1 + IGFBP1 cells stably overexpressing IGFBP1 were established using A549/SOX1 cells. Retrovirus particles, containing either IGFBP1 or empty vector, were produced in 293T producer cells. A549 cells were transduced with supernatants and selected with puromycin for 10 days. A549/SOX1 + IGFBP1 cells were established by collecting puromycin-resistant cells and confirmed by RT-PCR. Because SOX1 inhibits cell invasion but has no effect on cell proliferation, the invasion ability of IGFBP1 was directly tested, *in vivo*. To investigate the comprehensive function of SOX1 and IGFBP1, *in vivo*, A549 cells of different groups were injected intravenously into nude mice (five to six mice each group) via tail veins. All mice were euthanized for gross inspection and pathology inspection 10 weeks afterwards. Diagnoses of metastatic nodules were confirmed by H&E staining. In this model, overexpression of IGFBP1 counteracted the metastasis-inhibiting effects of SOX1 (**Figure 3C**). Notably, higher incidence of bone metastasis was observed in IGFBP1 overexpressing group compared to control group, determined by x-ray and histological analysis. It was found that bone metastasis occurred in A549/SOX1 + IGFBP1 cell-injected mice but not in A549/SOX1 cell-injected mice, by x-ray (3/6 vs 0/6, **Figure 3D**). This result indicates that IGFBP1 also plays an important role in the process of bone colonization during the secondary spread of human NSCLC cells, *in vivo*.

SOX1 inhibits angiogenesis via IGFBP1/MIF pathways in NSCLC

The above data indicates an important role of SOX1 in tumor angiogenesis of NSCLC, *in vitro* and *in vivo*, prompting this present study to investigate underlying molecular mechanisms. To further determine the roles of SOX1 and IGFBP1 in NSCLC tumor cell angiogenesis, the condition medium of A549/SOX1 and A549/SOX1 + IGFBP1 cells were collected and tube formation assays were performed. As a result, the culture medium of A549/SOX1 + IGFBP1 cells significantly promoted capillary tube formation of HUVECs (**Figure 4A**). It has been reported that IGFBP1 is a key mediator of MCSF (macrophage colony-stimulating factor)-induced angiogenesis. Therefore, whether overexpressed IGFBP1 contributes to tumor angiogenesis in NSCLC was investigated. To prove the hypothesis, experiments were carried out using supernatant from A-1/SOX1 and A549/SOX1 cells with IGFBP1 (250 ng/mL). This study found that inhibition of angiogenesis by SOX1 was reversed by subsequent treatment with IGFBP1 (**Figure 4B**). Many tumor-derived soluble factors are involved in the complex regulation of tumor angiogenesis. To determine which soluble factors mediated IGFBP1-induced tumor angiogenesis, secreted factors affected by IGFBP1 were analyzed using antibody arrays (R&D System, #ARY005B). A549 cells were cultured, with or without rhIGFBP1, for 48 hours before collecting the cell culture supernatant for antibody array analyses. As shown in **Figure 4A**, increased levels of MIF were detected in culture supernatant of A-1 cells treated with IGFBP1 compared with the addition of PBS negative control (**Figure 4C**). To confirm these findings, protein levels of MIF in supernatant were tested using enzyme-linked immunosorbent assay. It was found that protein levels of MIF in supernatant of A-1 and A549 cells were induced by the addition of rhIGFBP1 (**Figure 4D**). This study compared the angiogenesis ability of supernatant derived from rhIGFBP1-stimulated NSCLC cells, with or without pretreatment with MIF antibodies. Adding an MIF anti-body substantially inhibited the ability of supernatant, from NSCLC cells

treated with rhIGFBP1, to induce tube formation compared with the addition of IgG control antibodies (**Figure 4E**).

Discussion

Formation of new blood vessels is crucial for solid tumor growth and metastasis. Higher tumor microvessel density is associated with shorter survival in patients with lung cancer. Tumor cells actively release pro-angiogenic factors, such as vascular endothelial growth factor, to promote endothelial cell proliferation, survival, and migration for formation of new blood vessels. Lung cancer is the leading cause of cancer mortality worldwide. It accounts for over a million deaths, annually, and still has poor prognosis. In previous work, it was found that SOX1 silencing enhanced the cisplatin-mediated autophagy in NSCLC and promoter methylation of SOX1 was induced by long-term cisplatin treatment.

This present study found that ectopic expression of SOX1 could inhibit angiogenesis in both *in vitro* and *in vivo* conditions. Downregulated levels of SOX1 expression were inversely associated with MVD. It was found that overexpression of SOX1 in lung cancer cells inhibited expression of several genes, including CARS, TM4SF1, IGFBP1, CLDN1, STC2, and PDIA1, exhibiting mitogenic and pro-angiogenic activities. In addition, qRT-PCR analysis was performed with results showing that endogenous expression levels of CARS, TM4SF1, IGFBP1, CLDN1, STC2, and PDIA1 in A-1/SOX1 cells were all significantly reduced at the mRNA level. Among them, IGFBP1 was the most highly downregulated gene. IGFBP1 has been identified as a novel mediator of angiogenesis. Thus, IGFBP1 should be selected as a candidate target for further analyses.

To confirm IGFBP1 being targeted by SOX1, luciferase reporter containing the 2.0-kb region upstream of IGFBP1 gene was constructed. Luciferase activity assay showed that expression of the IGFBP1 reporter was significantly reduced by co-transfection with SOX1-overexpressing plasmid. It was also found that IGFBP1 upregulation counteracted SOX1-inhibited metastasis *in vivo*. Next, whether overexpressed IGFBP1 contributes to tumor angiogenesis in NSCLC was investigated. Agreeing with previous findings showing that IGFBP1 is a potent pro-angiogenic factor, this study showed that inhibition of angiogenesis by SOX1 was reversed by subsequent treatment with IGFBP1. To determine which soluble factors mediated IGFBP1-induced tumor angiogenesis, secreted factors affected by IGFBP1 were examined using antibody arrays. rhIGFBP1 transfection caused an increase in MIF levels in culture media of NSCLC cells. MIF has been identified as a target of IGFBP1. Secreted IGFBP1 not only promotes endothelial tube formation, in a paracrine manner, but also acts as an autocrine growth factor to increase expression of MIF. Kamimura et al. first showed that MIF expression in primary lung adenocarcinoma was related to patient prognosis [17]. Patients with hepatocellular carcinoma (HCC), having high expression levels of MIF, had a poor prognosis and MIF became an independent predictor of DFS. MIF is a proinflammatory cytokine shown to promote angiogenesis in several cancers, including bladder cancer, breast cancer, CRC, prostate cancer, and HCC [18-21]. It has been reported that exogenous MIF stimulates both VEGF and IL-8 expression in neuroblastoma cell lines, in a dose-dependent manner, and MIF-induced increases in these promoters of angiogenesis could be abrogated by the addition of a MIF monoclonal antibody [22]. Results of the present study showed that IGFBP1-induced MIF contributed to IGFBP1-induced angiogenesis in NSCLC. These findings agree with previous studies showing that MIF acts as a pro-angiogenic factor in NSCLC cells.

Taken together, these findings suggest that overexpression of SOX1 inhibits angiogenesis by direct regulation of IGFBP1/MIF pathways in NSCLC. The present data offers promising evidence that IGFBP1 may function as a pro-angiogenic factor in human NSCLC by promoting tumor angiogenesis via regulating MIF. Considering that anti-angiogenesis therapy is an important strategy of cancer treatment, SOX1 might be a novel therapeutic target for NSCLC.

Acknowledgements

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Disclosure of conflict of interest

None.

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