The HIF-1α/Sema4D axis promotes hypoxia-induced metastasis of human osteosarcoma via PI3K/Akt/mTOR signaling pathway

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Abstract

Purpose Semaphorin 4D (Sema4D) plays a vital role in varied tumor biological processes. Hypoxia inducible factor-1α (HIF-1α), an essential regulatory factor in tumor microenvironment, mediates hypoxia-induced-oncogenes overexpression during cancer occurrence and development. However, the function of Sema4D and its connection with HIF-1α are not entirely validated in osteosarcoma.

Methods RT-qPCR and immunohistochemistry were used to evaluate the differential expression of HIF-1α and Sema4D in human osteosarcoma. The biological function of Sema4D in osteosarcoma malignant phenotype was measured by RNA interference, Cell Counting Kit-8 (CCK-8) assay, Wound healing, Transwell and Tube formation assay. The stable transfected cells were established by lentivirus transfection to observe the biological significance of Sema4D in vivo. The relation between HIF-1α and Sema4D was explored through dual luciferase report assay.

Results This study showed HIF-1α and Sema4D expression levels were upregulated in human osteosarcoma samples and cell lines. Sema4D knockdown in 143B and MG63 cells restrained cell growth, migration and invasiveness via suppressing PI3K/Akt/mTOR signaling pathway. The tube formation capability of HUVEC was inhibited after coculture with low tumor-derived Sema4D. Besides, knockdown of Sema4D suppressed tumor growth in the xenograft tumor models. Moreover, Sema4D participated in the hypoxia-induced metastasis of osteosarcoma cells and its expression level was upregulated by transactivation of HIF-1α in vitro.

Conclusions Our results demonstrated that Sema4D knockdown had anti-osteosarcoma values and HIF-1α/Sema4D axis regulated the osteosarcoma metastatic cascade in hypoxia condition. They highlighted a therapeutic target which potentially offer an opportunity to improve the survival for osteosarcoma patients.

Introduction

Osteosarcoma (OS), the most common primary malignant bone tumor, exhibits an inclination to occur in children metaphysis and adolescent long bone (Ritter et al. 2010). At present, the main category for osteosarcoma treatment is neoadjuvant chemotherapy combined with surgery, which has improved the 5-year survival rate to approximate 70%. Despite this, therapies in osteosarcoma have been at a plateau in the past three decades as result of lung metastasis, which is the leading cause of fatal outcomes in recurrent osteosarcoma patients (Meazza et al. 2016; Harrison et al. 2018; Ferrari et al. 2015). Therefore, more research should be conducted to validate the underlying mechanisms of osteosarcoma invasion and metastasis.

Semaphorin family is constituted by more than 20 types of secreted or transmembrane semaphorin molecules containing a common conserved “Sema” domain (Neufeld et al. 2016; Battistini et al. 2016; Worzfeld et al. 2014). They are first identified as neural axon growth guiding factor during neural development and has roles in a range of biological processes such as cardiogenesis, angiogenesis, bone

remodeling, tumorigenesis and immune disorders. Semaphorin 4D (Sema4D), also called CD100, is a transmembrane homodimer belonging to class IV semaphorins and expressed in multiple cell types including immune cells, differentiating neurons and cancer cells (Ch'ng et al. 2010; Tamagnone et al. 2019). As a kind of transmembrane molecule, Sema4D can be cleaved under the effect of metalloproteinases (MMPs), releasing its soluble extracellular portion. Evidence is mounting that Sema4D is overexpressed in some malignances (HNSCC, NSCLC, ovarian cancer, breast cancer, colorectal carcinoma, etc.) and participated in tumor progression including angiogenesis, immune regulation and invasive growth (Lu et al. 2019; Yang et al. 2016; Suvannasankha et al. 2016; Liu et al. 2014; Soone et al. 2012). Ch'ng (Ch'ng et al. 2007) et al reported that Sema4D was an independent prognosticator for disease-free survival (DFS) of soft tissue sarcoma (STS). More importantly, Branden S (Moriarity et al. 2015) and his colleagues validated the high expression level of Sema4D in human osteosarcoma samples via RNA sequencing. These promising studies greatly interested us to further explore Sema4D in osteosarcoma.

The imbalance between rapid tumor growth consuming high oxygen and abnormal blood vessels with low oxygen results in hypoxia microenvironment, which is a typical character of solid tumor (Xu et al. 2016; Tafani et al. 2016; Seeber et al. 2011). Hypoxia-inducible factor-1 alpha (HIF-1α) is a heterodimeric transcription factor that is composed of two subunits and stabilized in hypoxia microenvironment to regulate multiple genes expression such as PDK-1, VEGF and CXCR4, which participate in tumor angiogenesis, invasion and treatment failure (Kim et al. 2006; Lu et al. 2009; Guan et al. 2015). The regulation role of hypoxia in Sema4D expression has already been described in HNSCC, bone metastasis in lung cancer (Sun et al. 2009; Chen et al. 2019). However, very little was known about the exact relationship between HIF-1α and Sema4D in human osteosarcoma.

In the current study, we examined that effect of Sema4D knockdown on osteosarcoma malignant phenotype in vitro and in vivo, as well as its connection with HIF-1α under hypoxia condition.

Materials And Methods

Materials

Antibodies used for this study were as follows: Ki-67(Cat# Ab16667), Sema4D (Cat# Ab134128) were from Abcam (Bratain); PI3K (Cat# AF6241) was from Affinity (USA); Phospho-PI3K (Cat# 310163), AKT (Cat# 10176-2-AP), Phospho-AKT (Cat# 66444-1-Ig) and E-Cadherin (Cat# 20874-1-AP) were from Proteintech (USA); mTOR (Cat# 380411), Phospho-mTOR (Cat# 385033), HIF-1α (Cat# 340462), CD34 (Cat# 380842), Vimentin (Cat# R22775), N-Cadherin (Cat# 380671) were from ZENBIO(China). Cocl2 (Cat# 232696) was from Sigma (USA).

Clinical human tissue specimen

Fourteen pathological tissues (seven pairs) were obtained from the osteosarcoma patients diagnosed at the Zhongnan Hospital of Wuhan University (Wuhan, China). The study was approved by Ethics and

Scientific Committee and the patients provided written informed consent before tissue acquisition. The clinical information of these patients was showed in Table S1.

Cell culture and hypoxia treatment

The human OS cell lines (143B, MG63, U2OS, HOS), human osteoblast cell line (hFOB) and 293T cells were obtained from American Type Culture Collection (Manassas, USA). 143B and HOS were cultured in MEM medium (Hyclone, USA), supplemented with 10% fetal bovine serum (FBS) (Gibco, USA). U2OS were cultured in Mycco'5A Medium (Procell, China) with 10% FBS. MG63 and 293T were cultured in high-glucose DMEM medium (Hyclone, USA) with 10% FBS. hFOB were cultured in DMEM/F12 medium (Gibco, USA) with 20% FBS. All the culture medium was supplemented with 100 μg/ml penicillin/streptomycin and the cells were kept in humidifed incubator (37 °C, 5% CO2). For the hypoxia treatment, cells were cultured in tri-gas incubator (MCO-170MUVL, Panasonic, Japan) (94% N2, 5% CO2, 1% O2) or treated with 100 μM Cobalt chloride (Cocl2).

Cell Transfection

The siRNA oligomers were synthesized by GenePharma (Suzhou, China) and their sequences were showed in Table S1. 143B and MG63 cells were seeded into six-well plates (200,000 cells/well) overnight and transfected with siRNA consequences by GP-Transfect-Mate (GenePharma, Suzhou, China). After 24-48 hours, cells were collected for subsequent tests. Stable cell lines with Sema4D knockdown were established via lentivirus transfection techniques and the specific methods have been described previously (Wu et al. 2019).

RNA Isolation and Quantitative Real-Time PCR

Total RNA was extracted from 143B and MG63 cells using Trizol (Vazyme, Cat# R401-01, China) reagent per the manufacturer's instruction, then were measured using a spectrometer (NanoDrop Technologies, USA). After cDNA synthesis, RT-qPCR was carried out with CFX Connect Detector instrument (Bio-Rad, USA) and the relative mRNA expression level was calculated by the $2^{-\Delta\Delta Ct}$ method. All the primer sequences were listed in Table S1.

Western Blotting

Radioimmune precipitation assay (RIPA) buffer containing PMSF and phosphorylated protease inhibitor at ratio of 100:1:1 was used to lyse samples for 30min. Then the supernatant was collected by centrifugation (12,000 rpm, 10 min). The concentration of protein was measured by BCA Kit (Beyotime, Cat# P0012, China). In total, 20 μg protein were separated by SDS-PAGE and transferred to the polyvinylidene difluoride (PVDF) membrane (Millipore, Cat# 05317, USA), then the membranes carrying proteins were blocked with protein free rapid blocking buffer (Epizyme, Cat# PS108P, China) for 30 min at room temperature. After blocking, the PVDF membranes were incubated with corresponding primary antibodies (dilution ratio 1:1000) at 4℃ overnight. Next day, the blots were incubated with secondary

antibodies (HRP-conjugated) for 1 h at room temperature. Finally, immunoreactive proteins were visualized by chemiluminescence reagent (Abisin, Cat# abs920, China).

Immunohistochemistry

Paraffin-embedded tissue samples were cut into consecutive 4mm-thick sections. After dewaxing and rehydration, slides were incubated in 3% hydrogen peroxide for the endogenous peroxidase activity blocking. Antigen repair was performed by microwave heating method and the slides were blocked with 5% goat serum albumin. Subsequently, they were incubated with primary antibodies (1:100 dilution) overnight in 4°C wet box. Next day, after PBS washing, the slides were incubated with HRP-conjugated secondary antibody for 20 minutes at 37°C. Immunoreactivity was detected using diaminobenzidine (DAB) chromogen and observed under microscope (Olympus, Tokyo Japan).

Cell Proliferation Assay

Cell proliferation ability was measured by CCK-8 assay. Briefly, 143B and MG63 cells were seeded at 1000 cells/well in 96-well plates and 10μL CCK-8 solution was added to each well every 24 hours. After 2h for 37°C incubation, the absorbance values were read at 450 nm using microplate reader (Molecular Devices, USA).

ELISA assay for soluble Sema4D

Osteosarcoma cells were plated in 6-well plates. After transfection or hypoxia treatment, the culture medium was discarded and 2 ml of serum-free medium was added into each well. The tumor condition medium (TCM) was collected after 24h and the soluble Sema4D concentration was determined by ELISA Kit (Mlbio, Cat# ml060350, Shanghai, China) per manufacturer's instructions.

Migration and Invasion Assay

For the Wound healing assay, osteosarcoma cells were plated in 6-well plates until approximately 90% cell confluent. We used 200μL pipette tips to standardized wound scratching in cell monolayers and unbounded cells were gently rinsed out with PBS. After growing in the 1%FBS medium for a further 24 or 36 hours, the wound area images were captured by microscope (Olympus, Japan) and the data were represented by wound-healing percentage. Transwell assay was used to evaluate the migration and invasion ability of osteosarcoma cells. 2×10^4 cells suspension was added into the upper chamber (Corning, Cat# 3422, USA) with or without Matrigel gel (BD, Cat# 356234, USA), while lower chamber with medium containing 15% FBS. After incubation for 24 h, cells attached to the lower surface were fixed with 4% paraformaldehyde and stained, then were counted under inverted phase-contrast microscope (Olympus, Japan).

Tube Formation Assay

Matrigel gel was placed at 4 °C overnight and cultured in 96-well plates (60μl/well) at 37 °C until complete coagulation. Human Umbilical Vein Endothelial Cells (HUVEC) was seeded in the 96-well plates pre-coated with gel at the density of 2×10^4 /well. After 5h, tube formation was imaged with the inverted phase-contrast microscope (Olympus, USA).

Dual-luciferase reporter assay

The dual-luciferase report system was constructed by GenePharma (Shanghai, China) including HIF-1α overexpression plasmid and PGL3-basic vector inserted with Sema4D core promoter. 143B and 293T cells were co-transfected with pGL3-Sema4D-luciferase, pRL-TK (Promega, USA) and HIF-1α siRNA or HIF-1α overexpression plasmid at approximately 30% density. Luciferase activity was examined using the dual-luciferase assay kit (Vazyme, Cat# DL101-01, China).

Animal Experiments

To validate the role of Sema4D on osteosarcoma in *vivo*,143B cells were infected with shRNA-Sema4D lentivirus or negative control shRNA-NC lentivirus. Six to eight-week-old male BALB/c nude mice were purchased from Charles River (USA) and raised in a standard laboratory environment at the Animal Experiment Center of Wuhan University. They were divided into three groups to receive injection with 100 μl of different 2×10^6 143B cells. The weights and tumor volumes ($volume=0.5\times L\times W^2$) were measured every three days. All the mice were humanely sacrificed four weeks later and tumors were removed, weighed and collected for follow-up experiments. Our animal studies were approved by the Animal Care and Use Committee of Wuhan University.

Statistical Analysis

All data was presented at least three independent experiments and analyzed using unpaired t-test and one-way ANOVA by GraphPad Prism 8.0. Statistically remarkable was indicated by $P < 0.05$.

Results

HIF-1α and Sema4D was highly expressed in human osteosarcoma

Initially, the mRNA and protein expression level of HIF-1α and Sema4D in human OS cell lines was presented in Fig. 1A-B. Compared with hFOB, the mRNA level of HIF-1α and Sema4D in osteosarcoma cells were upregulated. Consistently, the expression of HIF-1α and Sema4D were increased compared with those in the adjacent non-cancerous tissues (Fig. 1C-D). Immunohistochemistry analysis indicated that the positive expression rates of HIF-1α and Sema4D in osteosarcoma tissues were 100% (7/7) and 71.4% (5/7), respectively (Fig. 1D). Based on the data above, 143B and MG63 were selected for the subsequent analysis.

Sema4D knockdown inhibited cell proliferation, migration and invasion capacity of human osteosarcoma cell lines in vitro

RNA interference technology was used to knockdown Sema4D in 143B and MG63 cells and the interference efficiency of siRNA was validated by RT-qPCR and Western Blottingting (Fig. 2A-B). The CCK-8 assay results suggested that Sema4D deficiency inhibited 143B and MG63 cells proliferation (Fig. 2C). We further assessed whether Sema4D exhibited promoting effects on cell migration and invasion. Wound healing assay showed that Sema4D knockdown reduced the percentage of scratch healing in both 143B and MG63 (Fig. 2D-E). Similarly, the total number of cells which were fixed to the chamber coated Matrigel gel were notably decreased compared with the si-NC group after Sema4D silencing (Fig. 2F-G). Then we observed the changes in epithelial mesenchymal transition (EMT) and Western Blottingting demonstrated increasing protein levels of E-cadherin, while N-cadherin and Vimentin expression was decreased after Sema4D silencing (Fig. 2H).

Sema4D knockdown inhibited the pro-angiogenic capacity and blocked the PI3K/Akt/mTOR signaling pathway in osteosarcoma cells

To clarify the role of Sema4D in angiogenesis, we detected the content of soluble Sema4D (sSema4D) in TCM. The ELISA assay result showed the decline of sSema4D after silencing (Fig. 3A). After co-culture with different TCM, the numbers of tubes formed by HUVEC in si-NC group was significantly more than that in si-Sema4D group (Fig. 3B-C). As for the regulatory mechanisms of Sema4D biological activities in osteosarcoma, it was found that Sema4D knockdown downregulated the expression of PI3K/Akt/mTOR signal pathway related protein including p-PI3K, p-Akt and p-mTOR (Fig. 3D).

Knocking down Sema4D prevented osteosarcoma growth in vivo

To further validate these findings in *vivo*, 143B cells transfected with different lentivirus were injected into the right axilla of BALB/c nude mice. About four weeks after injection, significantly smaller tumor sizes (Fig. 4A-B) and lower tumor weights (Fig. 4C) were found in two Sema4D-depleted mice groups compared with sh-NC control group. Western Blottingting demonstrated the reduced protein level of Sema4D, CD34, Vimentin, N-cadherin, p-PI3K, p-Akt and p-MTOR after Sema4D knockdown, while E-Cadherin increased (Fig. 4D). Consistently, immunohistochemistry staining showed the similar results (Fig. 4E).

Hypoxia enhanced the invasion and migration of osteosarcoma cell lines via HIF-1α/Sema4D pathway

To query the influence of hypoxia on osteosarcoma cells' metastatic ability, 143B and MG63 cells were cultured with 1% O2 for 12h and 24 h respectively. Transwell assay results showed increased migration and invasion capabilities both in 143B and MG63 after hypoxic culture. These changes, however, were absent after Sema4D knockdown (Fig. 5A-5C). In addition, the change of EMT-related and PI3K/Akt/mTOR downstream molecules coincided with changes in metastatic capacity (Fig. 5D). -

Sema4D expression was regulated by HIF-1α

To probe the relationship between HIF-1α and Sema4D, Cocl2 and 1% O2 treatment was used to induce hypoxia condition for subsequent experiments. As expected, it was shown that the variation of HIF-1α and Sema4D was synchronous in 143B and MG63 cells (Fig. 6A-B). We next constructed a dual-luciferase

reporter system to explore the specific regulatory mechanism and the results demonstrated the luciferase activity was weakened after HIF-1α knockdown, while increased after HIF-1α expression (Fig. 6C-D).

Discussion

The rapid growth and metastasis is the leading cause of osteosarcoma patients death. Therefore, it's urgent to reveal the underlying proliferation and metastasis mechanism of osteosarcoma. Although originally identified as axonal navigation signaling molecular, Sema4D is actually involved in a wide spectrum of physiological and pathological processes (Ch'ng et al. 2010; Lontos et al. 2018). Dysregulated expression of Sema4D was reported in multiple cancers and closely associated with the patients poor prognosis (Mastrantonio et al. 2021). In cervial cancer and colorectal cancer, Sema4D promoted lymphangiogenesis and tumor cell migration (Ikeya et al. 2016; Liu et al. 2014). In ovarian cancer, The increase of Sema4D expression was related to higher malignancy of epithelial ovarian carcinomas (Chen et al. 2012). More importantly, the role of Sema4D was complicated in HNSCC. Tumor cells-derived Sema4D promorted tumor growth and vascularity, as well as assisted immune escape by inducing MDSCs expansion (Younis et al. 2016). Rania H and his colleagues (Younis et al. 2021) revealed that most HNSCC patients had higher sSema4D level in plasma, which correlated to the histological immune excluded subtype. With regard of distant metastasis, Sema4D enhanced skeletal metastasis and represent a new therapeutic target in breast cancer and lung cancer (Yang et al. 2016; Chen et al. 2019).

Our studies suggested similar conclusions in osteosarcoma cells. Sema4D was overexpressed in human osteosarcoma samples and cell lines. Moreover, Sema4D silencing inhibited cell proliferation, migration and invasion simultaneously accompanied by the expression changes of cell EMT associated proteins. The PI3K/AKT/mTOR pathway was a critical driver of tumorigenesis and Sema4D promoted bladder cancer cells proliferation ability and metastasis by activating the PI3K/AKT pathway (Lu et al. 2019). In coincidence with these, our results clearly showed decreased protein level of p-PI3K, p-Akt and p-mTOR in osteosarcoma cells after Sema4D knockdown. PlexinB1, high affinity receptor of Sema4D, expressed mainly on endothelial cells. sSema4D released in stroma was involved in vascularity through binding to PlexinB1, subsequently activating RhoA and c-Met signaling pathway (Lontos et al. 2018; Ch'ng et al. 2010). In pancreatic cancer, inhibition of Sema4D expression significantly suppressed the angiogenic mimicry formation led by cancer cells (Xu et al. 2014). Besides, Sema4D expression in colorectal cancer was related with lymphatic infiltration and vascular invasion (Ding et al. 2016). Consistently, we founded a noticeable decrease of tube formation capability in HUVEC after cocultivation with TCM originating from Sema4D deficient osteosarcoma cells.

In the hypoxia microenvironment, a series of adaptive changes occurred in tumor cells including vascular growth factors secretion, oncogenes upregulation and intracellular signaling pathways activitation. As a dominate effector of the cellular response to hypoxia, HIF-1α has been previously explored in many solid tumors. There were numerous known downstream target genes of HIF-1α including GLUT1, PDK-1 and VEGF, which were associated with tumor angiogenesis, metastasis and metabolic reprogramming. In osteosarcoma, HIF-1α has been previously detected as a major regulator. Cao (Cao et al. 2020) et al.

reported that hypoxia-Induced WSB1 promoted the metastatic potential of KHOS/NP cells. Besides, HIF-1α/CXCR4 axis played a crucial role during osteosarcoma migration and might represent a novel therapeutic strategy (Guan et al. 2015). The role of the HIF-1α/Sema4D pathway in solid tumors has recently become a field of increasing interest. Chen (Chen et al. 2019) et al. highlighted its regulatory function in bone metastases of lung cancer and the similar findings was reported in HNSCC and Ovarian epithelial cancer (Zhou et al. 2012; Sun et al. 2009). In order to clarify the effect of HIF-1α/Sema4D pathway in osteosarcoma, we investigated the influence of Sema4D knockdown on hypoxia-induced osteosarcoma metastasis. Our results revealed Sema4D was the part of the hypoxia adaptive response because of the increased metastatic potential of 143B and MG63 cells under hypoxia treatment. This change, however, was not observed after Sema4D silencing. Even more critical evidence was the dual luciferase assay report clearly defined that HIF-1α exerted its transcriptional transactivation via directly binding to hypoxia response element (HRE) of the Sema4D promoter.

Several limitations existed in our studies. Firstly, more human samples should be collected for further analysis of the association between Sema4D expression level and clinicopathological characteristics. In addition, transgenic mice and anti-Sema4D antibodies such as VX15/2503 (Patnaik et al. 2016), which was validated in clinical trials, would be an ideal model *in vivo* experiment. Moreover, Sema4D was expressed in various types of tumor cells, not just in tumor cells, and its diverse roles in osteosarcoma progression in a hypoxic condition required further exploration.

In conclusion, our integrated approach revealed that HIF-1α and Sema4D expression was up-regulated in human osteosarcoma. Moreover, the knockdown of Sema4D suppressed osteosarcoma progression through PI3K/Akt/mTOR signaling pathway inactivation. In addition, HIF-1α activated Sema4D translational expression in osteosarcoma via binding to the HRE. Therefore, the HIF-1α/Sema4D axis can be viewed as an attractive target with promising therapeutic potential for osteosarcoma.

Declarations

Acknowledgements

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Author contributions

YW and JL provided study design and critical revision of the manuscript. YL, BN, TX and DW performed the experiments; YL and MWcollected the clinical samples; YL analyzed the data and wrote the manuscript until final submission.

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Data availability

All the data involved in this study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest

The authors declare no competing interest exits.

Ethics approval

The protocol obtained review and approval from the Ethics and Scientific Committee of Zhongnan Hospital of Wuhan University.

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Figures

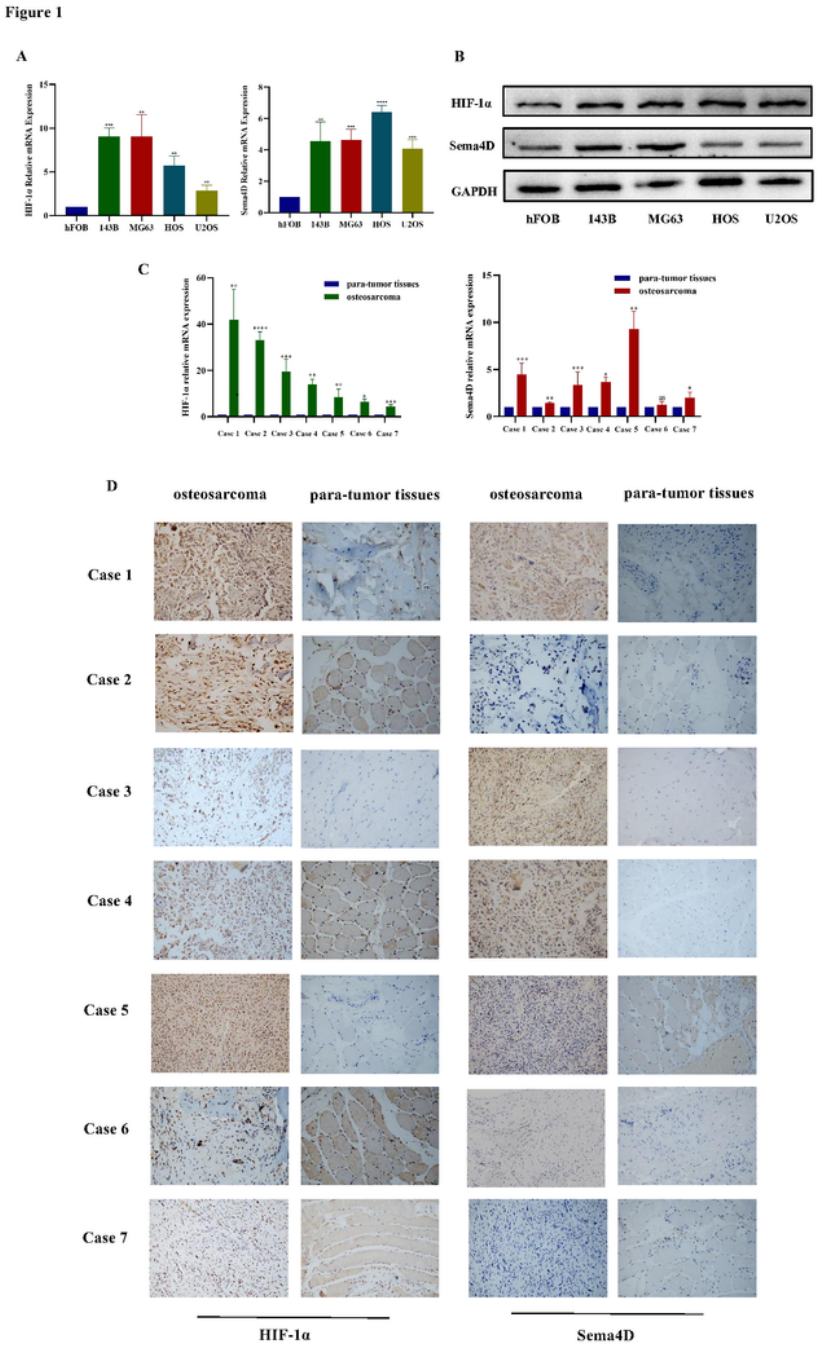

Figure 1

The expression levels of HIF-1α and Sema4D in human osteosarcoma. The mRNA (a) and protein (b) expression levels of HIF-1α and Sema4D in human osteosarcoma cell lines were higher than that in hFOB; The HIF-1α and Sema4D mRNA expression levels (c) and immunoreactivity (d) in osteosarcoma tissues

were upregulated compared with adjacent non-cancerous tissues. Seven clinically diagnosed osteosarcoma patients were measured by RT-qPCR and Western Blotting analysis. Representative images were shown (20×). *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001.

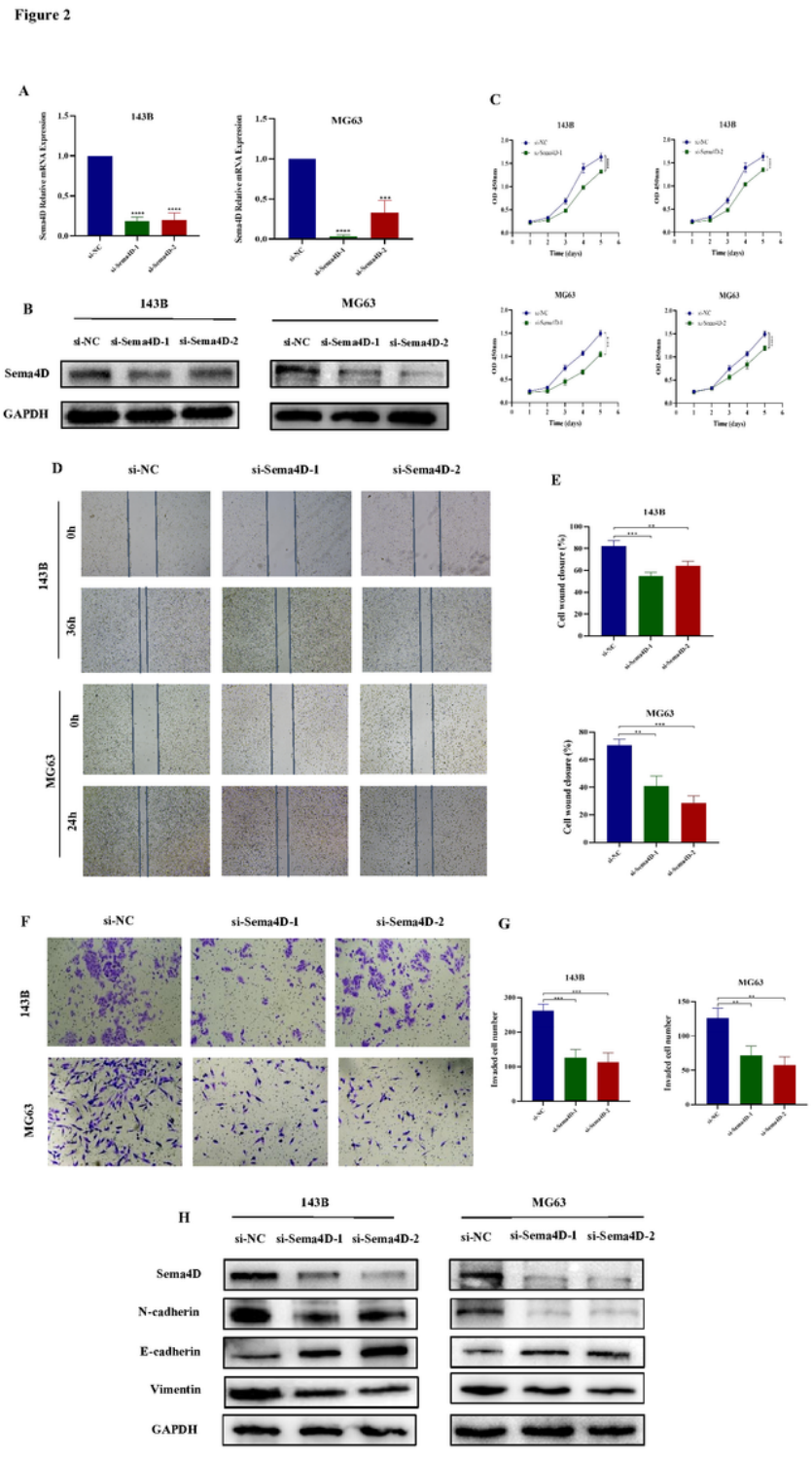

Figure 2

Inhibition of Sema4D decreased cell proliferation, migration and invasion in 143B and MG63 cells.

(a, b) Detection of transfection efficiency by RT-qPCR and Western Blotting, GAPDH was used as an internal control; (c) CCK8 assay revealed that Sema4D knockdown significantly inhibited 143B and MG63 cell proliferation; (d, e) Cell migration capacity was measured by wound-healing assay; (f, g) Cell invasion ability was measured by Transwell assay; (h) Immunoblotting of the EMT-related proteins. GAPDH was used as an internal control. Magnification: 10 ×. The results of three independent experiments were shown. ***p*<0.01, ****p*<0.001, ****p*<0.0001.

Figure 3

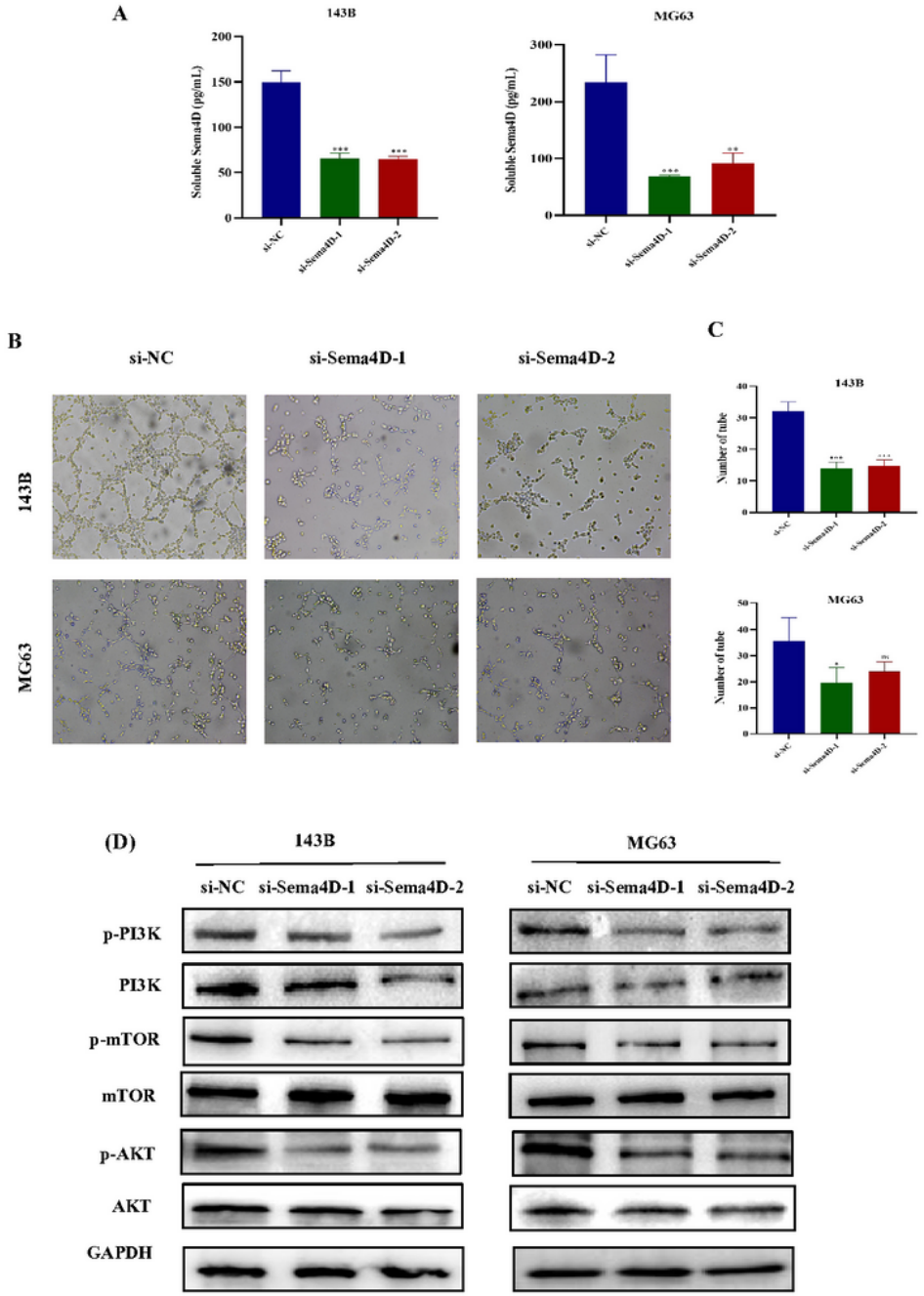

Figure 3

Sema4D knockdown inhibited angiogenesis and blocked the PI3K/Akt/mTOR signaling pathway in 143B and MG63 cells.

(a) The levels of secreted Sema4D in TCM from osteosarcoma cells were assessed using ELISA assay. (b, c) The tube formation capacity of HUVEC was inhibited after co-culture with TCM containing lower Sema4D; (d) Representative Western Blotting images of PI3K/Akt/mTOR signaling pathway related proteins, GAPDH was used as an internal control.

Magnification: 10 ×. The results of three independent experiments were shown. *p<0.05, **p<0.01, ***p<0.001.

Figure 4

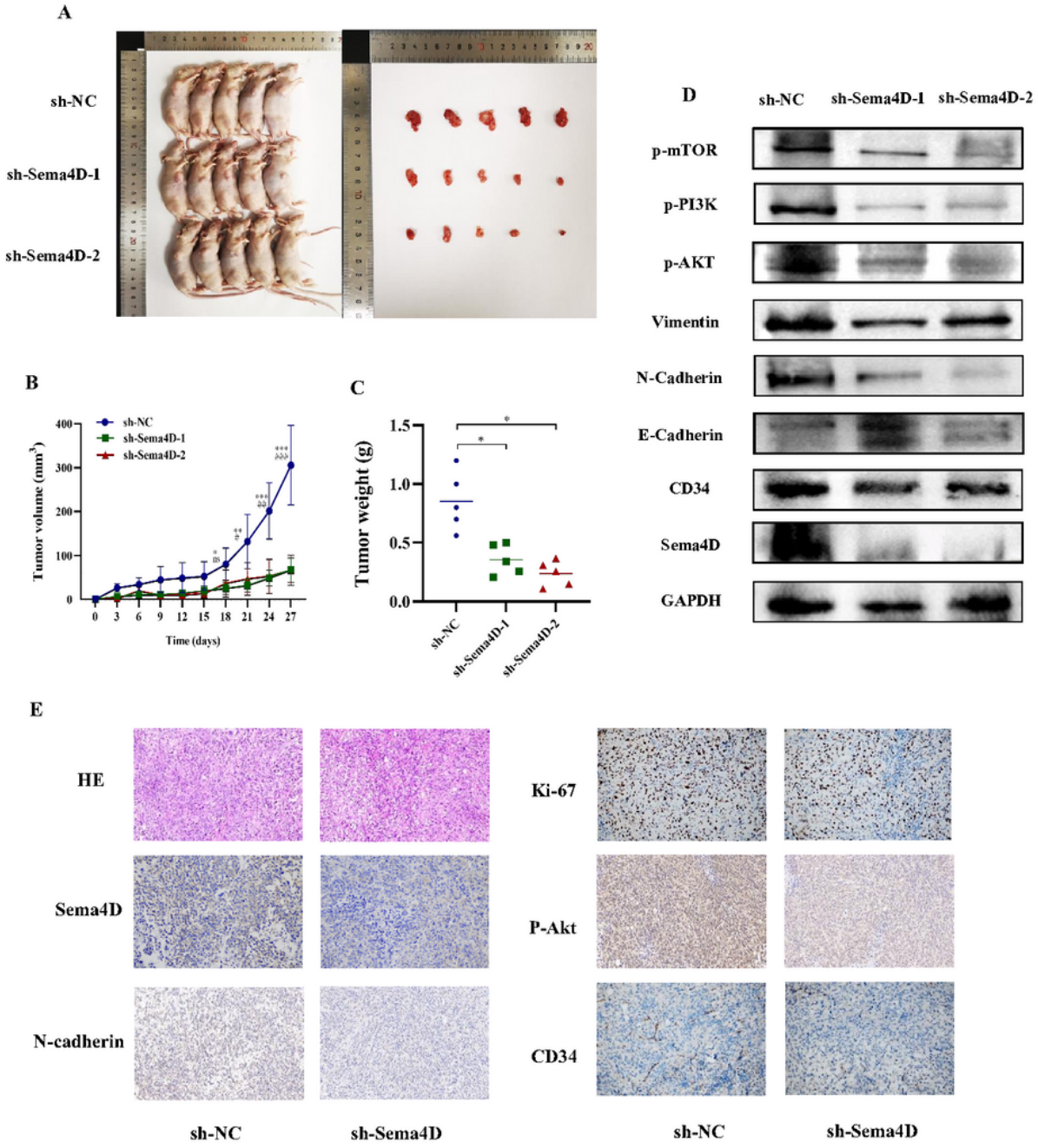

Figure 4

Knockdown of Sema4D significantly suppressed tumorigenesis in vivo

(a) Representative images of nude mice and xenograft tumors (n=5); (b) Tumor volume curve of xenograft tumors (n=5); (c) The tumor weight of xenograft tumors (n=5); (d) The Western Blotting results of mice xenograft tumor protein, GAPDH was used as an internal control. (e) H&E analysis and

immunohistochemical images of the Sema4D, Ki-67, CD34, N-Cadherin and p-Akt expressions in 143B xenograft tumors. Magnification: 20 ×. *p<0.05, **p<0.01, ***p<0.001.

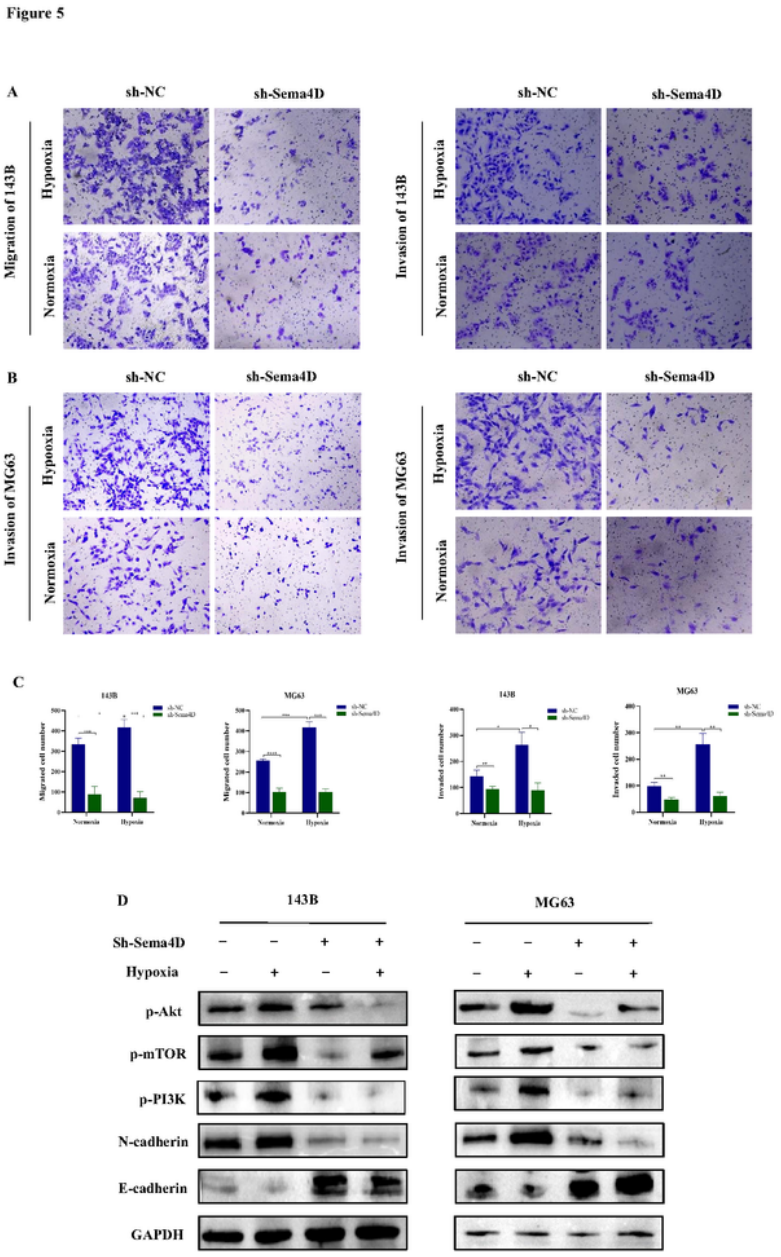

Figure 5

Sema4D knockdown blocked the hypoxia-induced migration and invasion in 143B and MG63 cells.

143B and MG63 cells, transfected with sh-NC or sh-Sema4D, were exposed to either normoxia or hypoxia condition; (a, b) Cell migration and invasion capability were measured by Transwell assay; (c) Quantifcation of the numbers of stained cells; (d) The Western Blotting results of 143B and MG63 cells with different treatments. Magnification: 20 ×. *p<0.05, **p<0.01, ***p<0.001, ***p<0.0001.

Figure 6

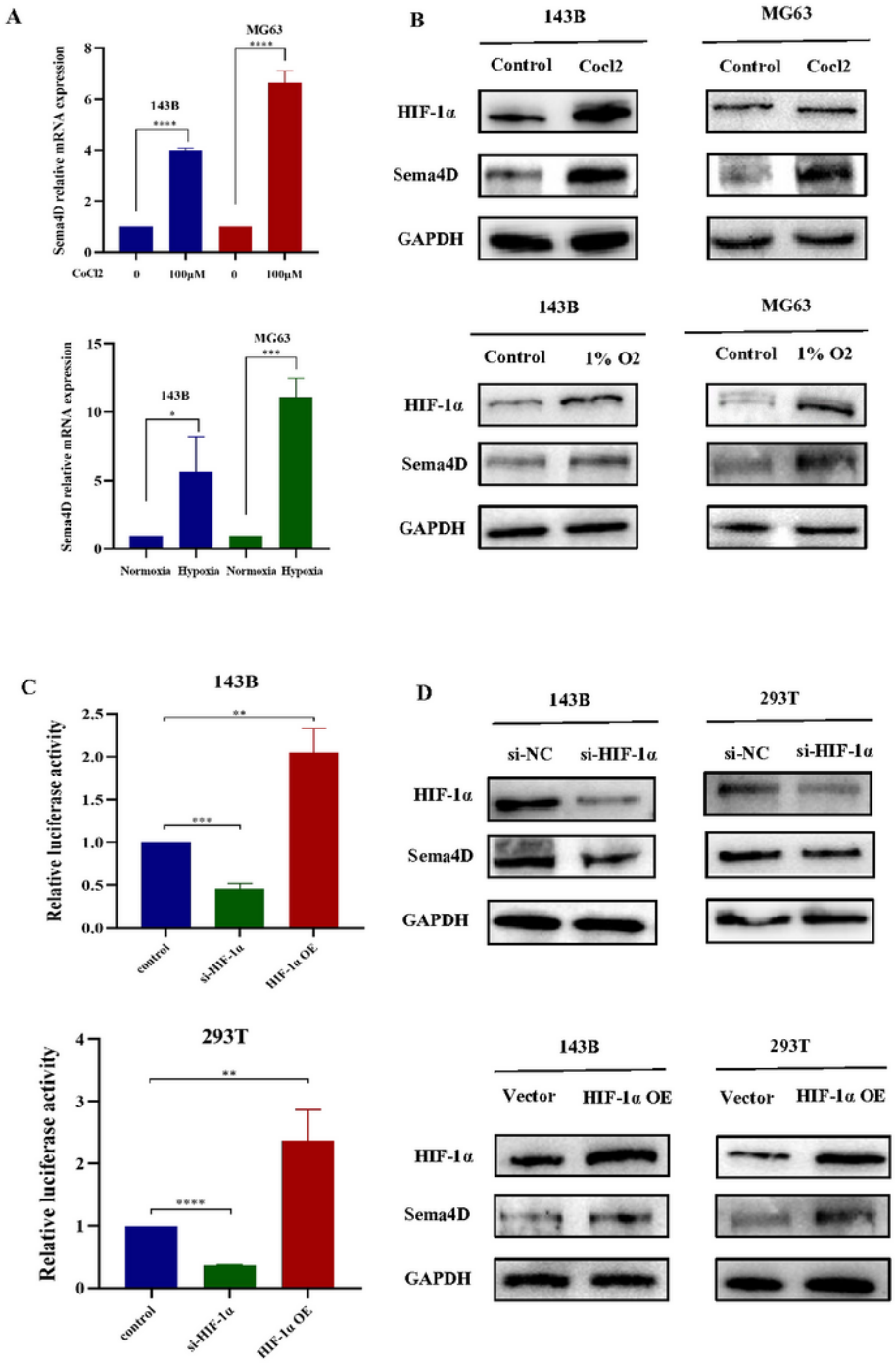

Figure 6

Hypoxia significantly increased Sema4D expression in a HIF-1α-dependent manner. (a, b) The mRNA and protein expression levels of HIF-1α and Sema4D were upregulated in 143B and MG63 after hypoxia treatment (100μM Cocl2 and 1% O2); (c) The dual luciferase reporter assay demonstrated a targeted relationship between HIF-1α and Sema4D in 143B and 293T cells; (d) Detection of transfection efficiency by RT-qPCR and Western Blotting, GAPDH was used as an internal control.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- TableS1.xlsx