miR-3156-5p Enhances Hypoxia-Induced Angiogenesis in Hepatocellular Carcinoma via Modulating the SOCS5/HIF-1α/VEGF Pathway

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Abstract

Background: MicroRNAs (miRNAs) are dysregulated in hypoxia-induced hepatocellular carcinoma (HCC). This study probed the regulatory mechanism of miR-3156-5p on HCC under hypoxia.

Methods: HCC cells (HepG2) were exposed to normoxia or hypoxia, and the conditioned medium (CM) of HepG2 was applied. Quantitative reverse transcription PCR (qRT-PCR) was implemented to analyze the miR-3156-5p profile. The cell counting kit-8 (CCK-8) assay and the colony formation experiment were conducted to measure cell proliferation, colony formation, and angiogenesis.

Results: The results manifested that miR-3156-5p was up-regulated in HCC cells and endothelial cells under hypoxia, and up-regulating miR-3156-5p boosted HCC cell proliferation, endothelial cell angiogenesis, and HIF-1α/VEGF expression.

Conclusions: miR-3156-5p activates the HIF-1α/VEGF pathway by hampering SOCS5, thereby enhancing the angiogenic potential of hypoxia-induced endothelial cells in HCC cells.

1. Background

Primary hepatocellular carcinoma, mainly caused by chronic infection with hepatitis B virus (HBV) and hepatitis C virus (HCV), is the second leading cause of cancer-associated deaths worldwide [1, 2]. Transcranial arterial embolization or chemical embolization (TAE/TACE) is a treatment option for advanced hepatocellular carcinoma (HCC). However, TACE or TAE can cause hypoxia and contribute to the formation of the vascular system through synergistic correlation with other angiogenic factors, such as vascular endothelial growth factor (VEGF), so as to enhance tumor growth [3–5]. Therefore, understanding the mechanism of angiogenesis in tumors and developing methods to repress tumor angiogenesis are novel therapies for tumor inhibition.

MicroRNAs (miRNAs) are highly conserved small non-coding RNAs about 22 nucleotides long, which are implicated in post-transcriptional regulation of gene expression [6]. Recent studies have demonstrated that miRNAs can interact with target mRNAs and participate in cell metastasis and angiogenesis [7]. For example, miR-140-5p restrains VEGF-A expression and dampens angiogenesis, cell viability, colony formation, and invasion [8]. miR-210 directly targets and curbs fibroblast growth factor receptor 1 (FGFRL1) expression to boost hypoxia-induced HCC angiogenesis [9]. Also, miR-182 is distinctly up-regulated in hypoxia-induced HCC tissues, and it motivates HCC angiogenesis by curbing RASA1 [10]. miR-3156-5p, a miRNA, is up-regulated in breast cancer, and its expression attenuation impedes cell proliferation and sensitizes cells to chemotherapy [11]. Nevertheless, the function of miR-3156-5p in HCC remains unknown.

The suppressor of cytokine signaling (SOCS) family is an essential negative regulator of cytokine signaling, and dysregulation of SOCS is associated with diversified cancers [12]. SOCS5, a member of the SOCS family, is overexpressed in HCC tissues, and SOCS5 overexpression heightens *in-vitro* HCC cell

migration and invasion through inactivation of PI3K/Akt/mTOR-mediated autophagy [13]. Additionally, Bi HQ et al. found that SOCS5 was down-regulated in HCC cells, and its up-regulation restrained tumor cells' proliferation [14]. These reports confirmed that that SOCS5 was aberrantly expressed in HCC and could exert carcinogenic or tumor-suppressive roles. On the other hand, classical theories hold that miRNAs regulate gene expression at the post-transcriptional level by attenuating messenger RNA (mRNA) translation or boosting mRNA degradation. For example, miR-9-5p affects the angiogenesis and radiosensitivity of cervical cancer cells by targeting SOCS5 [15]. Nevertheless, the mechanism of miR-3156-5p and SOCS5 in HCC remains elusive.

Hypoxia is a common cause of multiple malignancies, and hypoxia contributes to angiogenesis by stimulating the hypoxic-inducible factor-1α (HIF-1α)/VEGF axis [16]. Liu K et al. showed that HIF-1α and VEGF expression in the serum of HCC patients was elevated after TACE [17]. Besides, inhibiting the HIF-1α/VEGF pathway under hypoxia chokes HCC tumorigenicity and angiogenesis [18, 19]. Thus, inhibiting the HIF-1α/VEGF pathway has guiding significance for the treatment of HCC.

Here, we hypothesized that miR-3156-5p contributed to HCC by regulating HCC cells' angiogenesis under hypoxia. Therefore, we tested the angiogenic potential of endothelial cells transfected with miR-3156-5p mimics under hypoxia. It was found that overexpressing miR-3156-5p up-regulated the HIF-1α/VEGF pathway by attenuating SOCS5, thereby heightening the proliferation and invasion of HCC cells and facilitating the angiogenesis of endothelial cells. These findings further reveal the correlation between miR-3156-5p and HCC.

2. Methods And Materials

2.1 Cell culture

The human HCC cell line (HepG2) was bought from the Cell Center of the Chinese Academy of Sciences (Shanghai, China). Cells were cultured in the Dulbecco modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Gibco) and penicillin/streptomycin (Sigma, St.Louis, MO, USA) under 37°C/5% CO_2 hypoxia (1% O_2) or normoxia (21% O_2).

Human umbilical vein endothelial cells (HUVECs) were purchased from Allcells (Shanghai, China) and cultured in the medium containing 50 U/mL penicillin and 50 U/mL streptomycin (Invitgen, San Diego, CA), 50 mL endothelial cell growth additive (Upstate, Temecula, CA), 2.2 g/L sodium bicarbonate, and 10% fetal bovine serum (Gibco, Grand Island, NY) under a humid environment at 37˚C with 5% CO_2 [19].

2.2 Cell transfection

The pcDNA empty vector (NC), pcDNA-SOCS5 (SOCS5), miRNA control (miR-NC), miR-3156-5p mimics, miR-NC-in, and miR-3156-5p inhibitors were purchased from GenePharma Co., Ltd. (Shanghai, China). HepG2 cells were inoculated on 24-well cell plates (3×10^5 cells/well). After incubation at 37˚C with 5% CO_2 for 24 hours, the cells were transfected using Lipofectamine® 3000 (Invitrogen;

ThermoFisherScientific, Inc.). The transfection validity was measured via quantitative reverse transcription PCR (qRT-PCR). The cells were incubated at 37°C with 5% CO_2 for 24 hours for further analysis.

2.3 Collection of conditioned medium (CM)

As mentioned previously [21], HepG2 cells overexpressing or knocking down miR-3156-5p were cultured in a normal growth medium (DMEM + 10% FBS + 1% penicillin/streptomycin) to 80% fusion. The cells were then washed twice with PBS and starved overnight in a serum-free medium (DMEM). Subsequently, they were treated with hypoxia (1% O_2) for 12 hours. Thereafter, the culture supernatant was collected, centrifuged at 16000 rpm for 5 min to remove the cell debris, and stored at -80°C.

2.4 Cell counting kit-8 (CCK-8) assay

HepG2 cells were sub-cultured in 96-well plates for 24 hours. Next, they were randomly divided into the miR-NC group, the miR-3156-5p group, the NC-in group, and the miR-3156-5p-in group. After cell transfection, 10 μL CCK-8 reagent (Dojindo Molecular Technologies, Kumamoto, Japan) was added to each well according to the kit's instructions and further incubated for two hours. The absorbance was observed with a microplate reader to calculate cell growth rate (proliferation ratio = cell absorbance/ 0 hour absorbance).

2.5 BrdU assay

Cell proliferation was examined with the 5-Bromo-2-deoxyUridine (BrdU) method. Briefly, the transfected HCC cells were inoculated in 96-well plates and cultured for 24 hours. The BrdU labeling reagent (Wuhan AmyJet Scientific Inc. Wuhan, China) was added according to sigma's instructions, and the plates were incubated with 5% CO_2 at 37℃. After 48-hour incubation, immunofluorescence staining of cells was performed following the BrdU antibody operating instructions. The positive cell number and the total DAPI-positive cell number under the microscope were randomly chosen from three fields of view and counted. Cell proliferation rate = BrdU-positive cell number/DAPI-positive cell number, and the average value was taken.

2.6 Tube formation experiment *in vitro*

Endothelial cells were incubated at 37°C for 6 hours with CM of anoxic HepG2 cells in Matrigel matrix (BD Biosciences)-coated 24-well plates (1×10^4 cells/well). Images were then taken under the microscope, and analysis was performed with the Image Pro-Plus 6.0 software (National Institutes of Health, ×100). The tube length was measured [22].

2.7 Western blot (WB)

Mouse tumor tissues were collected and each 100 mg of tissue was lysed by 1 mL of pre-cooled tissue lysis solution. Subsequently, the tissues were subjected to homogenization by ultrasonic crusher (ice bath) and centrifugation for supernatant collection. The cells were retained, incubated with pre-cooled RIPA lysate (Beyotime Biotcchnology, Shanghai, China) on ice for 20 min, and centrifuged at 13000 rpm

at 4°C and for 20 min. Then, the supernatant was removed, and the protein quantification of mouse tumor tissues and cells was made with the BCA protein quantification kit. Afterward, the proteins were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA, USA). After being blocked with 5% skim milk, the membranes were incubated with the primary antibodies (Abcam, MA, USA; 1:1000) of SOCS5 (ab97283), anti-HIF-1α (ab179483), anti-VEGF-A (ab52917), anti-JAK2 (ab108596), anti-p-JAK2 (ab32101), anti-STAT3 (ab68153), anti-p-STAT3 (ab267373), and anti-β-actin (ab227387) at 4°C. The membranes were cleaned three times the next day. Then, the TBST-diluted secondary antibody was added and incubated for one hour on a shaker at room temperature. Next, the membranes underwent three times of washing. The ECL chemiluminescence reagent (Amersham Pharmacia Biotech, Little Chalfont, UK) was tested in a gel scan analyzer and the results were analyzed. ImageJ was utilized to analyze the grayscale values of each band, and the ratio of the grayscale value of the target protein to the grayscale value of GAPDH served as the relative protein expression for analysis.

2.8 qRT-PCR

Total RNA was extracted from mouse tumor tissues and HepG2 cells with the TRIzol reagent. cDNA synthesis was implemented with the miScript II Reverse Transcription Kit (Qiagen, Hilden, Germany) or PrimerScript RT Kit (Takara Bio, Inc., Otsu, Japan) for miRNA and mRNA detection. The PCR reaction mixture (20 μL) was placed into a LightCycler 480 II real-time PCR instrument (Roche Diagnostics, Basel, Switzerland) and a miScript SYBR Green PCR kit (QIAGEN, Dusseldorf, Germany) to test miRNA or mRNA.The PCR conditions were as follows: predenaturation for 5 min at 95˚C, denaturation for 15 s at 95˚C, and annealing for 30 s at 60˚C. U6 was the endogenous control of miR-3156-5p, while GAPDH was that of HIF-1α and VEGFA, with the $2^{(-\Delta\Delta Ct)}$ method for statistics. Each experiment was conducted three times. Specific primer sequences are as follows:

The target	Forward (5 '-3')	Reverse (5 '-3')
miR-3156-5p	GGTACCGCCGGGAGGGTCTGCC	CTCGAGTTCAATTTAACAACAAATTGCAAA
HIF-1α	AAGTCTGCAACATGGAAGGTAT	TGAGGAATGGGTTCACAAATC
VEGF-A	AAGGAGGAGGGCAGAATCAT	ATCTGCATGGTGATGTTGGA
U6	CGCTTCGGCAGCACATATAC	TTCACGAATTTGCGTGTCAT
GAPDH	GCTCTCTGCTCCTCCTGTTC	ACGACCAAATCCGTTGACTC

2.9 Dual-luciferase reporter assay

All luciferase reporting vectors (SOCS5-1-WT and SOCS5-1-MUT) were constructed by Promega (Madison, WI, USA). HepG2 cells (4.5×10^4) were inoculated in 48-well plates and cultured to 70% confluence. Then, they were co-transfected with SOCS5-1-WT, SOCS5-1-MUT and miR-3156-5p mimics or negative controls by Lipofectamine 2000. The luciferase activity was determined as per the manufacturer's instructions 48 hours after the transfection. We conducted all experiments three times.

2.10 RNA immunoprecipitation (RIP)

RIP was performed with the Magna RIP Kit (EMD Millipore, Billerica, MA, United States). After the cells were lysed with RIP buffer, the AgO-2 antibody (micropores) or the control antibody (normal mouse immunoglobulin, micropores) was added. After incubation overnight at 4˚C, the adsorbed lysates were retained, and the RNA was extracted. The SOCS5 expression was tested by qRT-PCR.

2.11 The xenograft model in nude mice

Twenty 5-week-old female BALB/c nude mice weighing 180–200 g were purchased from the Animal Experiment Center of Lanzhou University. The mice were kept in cages at 20–25℃ with 50%-52% humidity, with a light-dark cycle of 12 hours each. The mice could drink and eat at will. All experiments were authorized by the Ethics Committee of the First Hospital of Lanzhou University and were in line with the Guidelines of the National Institutes of Health on animal care and use. 2×10^6 HepG2 cells transfected with miR-3156-5p and the negative controls were injected into both armpits of the mice subcutaneously (n = 5), respectively, and tumor growth was observed. Tumor volume was measured 4, 8, 16, 24, and 32 days after the injection. The mice were killed on the 32nd day, and tumor volume (mm^3) and weight (g) were determined. Tumor volume was calculated according to the following formula: volume = length × $width^2$×0.5.

2.12 Immunohistochemistry (IHC)

After conventional paraffin embedding and sectioning (4 μM), the xenograft tumors were dewaxed with xylene and hydrated with gradient alcohol. The endogenous peroxidase was inactivated by blocking with 3% H_2O_2 for 10 min. Microwave repair was made with 0.01 mol/L sodium citrate buffer (pH = 6.0, 15 min). After blocking with 5% bovine serum albumin (BSA) for 20 min, the primary antibodies of SOCS5 (1:200), p-STAT3 (1:200), HIF-1α (1:200), and VEGFA (1:200) were added and incubated at 4˚C overnight. The next day, the goat anti-rabbit secondary antibody was added and incubated for 20 min at room temperature. Then, the sections were washed with PBS and visualized with DAB. After hematoxylin counterstaining, the sections were dehydrated and transparentized for mounting inspection.

2.13 Statistical analysis

T test was employed to compare the two groups of data, and the differences between multiple sets of data were compared by one-way analysis of variance. The differences between the two sets of data were analyzed by the post hoc test, and SPSS24.0 (SPSS Inc., Chicago, Illinois, USA) was employed to calculate the Mean ± SEM results in the experiment. The GraphPad 8.0 software was adopted for mapping, and $P < 0.05$ presented statistical significance.

3. Results

3.1 miR-3156-5p, HIF-1α and VEGFA were up-regulated in HCC cells and endothelial cells

HCC cells and hypoxia-induced HCC cells were employed to perform microarray. Fold change > 2 and $P < 0.05$ were applied to explore the abnormal miRNA expression (data from the GEO database, GEO accession number: GSE68593). As indicated by the volcanic plot and the heat map, miR-3156-5p was highly expressed in hypoxia-treated HCC cells by contrast with the HCC cells in normal group (log_2(fold change) = 4.85, p = 3.06E-09, Fig. 1A, B). Hence, in the next experiments, we focused on the expression and effects of miR-3156-5p in HCC cells. We cultured HepG2 cells under hypoxia (1% O_2) and normoxia (21% O_2) and monitored the expression of miR-3156-5p, HIF-1α and VEGFA by qRT-PCR. As a result, miR-3156-5p, HIF-1α, and VEGFA were up-regulated in hypoxia-induced HCC cells compared with those treated under normoxic conditions (Fig. 1C-E). The CM of HepG2 cells was harvested to culture HUVECs. Then, the expression of miR-3156-5p, HIF-1α and VEGFA in HUVECs was compared by qRT-PCR. Similarly, the miR-3156-5p, HIF-1α, and VEGFA were all up-regulated in hypoxia-induced HUVECs (Fig. 1F-H). Therefore, miR-3156-5p might be involved in HCC progression derived by hypoxia.

3.2 miR-3156-5p heightened HCC cell proliferation and facilitated tube formation of endothelial cells

The above studies illustrated that miR-3156-5p was highly expressed in HCC cells. Next, we evaluated the effects of high and low expression of miR-3156-5p on HCC cells and endothelial cells by gauging the proliferation of HepG2 cells and tube formation of HUVEC cells. miR-3156-5p mimics, inhibitors and their corresponding negative controls were transfected in HepG2 cells. qRT-PCR illustrated that miR-3156-5p was highly expressed in HepG2 cells after transfection of miR-3156-5p mimics (vs. the con. group). By contrast with the miR-in group, transfection of miR-3156-5p inhibitors in HepG2 cells resulted in low miR-3156-5p expression (Fig. 2A). Later, CCK-8 and BrdU experiments confirmed that up-regulating miR-3156-5p motivated HepG2 cell proliferation, while down-regulating miR-3156-5p exerted the reverse effect (Fig. 2B, C). The CM of HepG2 cells was collected and co-cultured with HUVECs for 24 hours (Fig. 2D). The *in-vitro* tube formation assay showed that angiogenesis of HUVEC was boosted by miR-3156-5p up-regulation and impeded by miR-3156-5p down-regulation (Fig. 2E). In addition, qRT-PCR and WB results testified that mRNA and protein expression of HIF-1α and VEGFA were elevated in HepG2 cells and HUVECs after transfection of miR-3156-5p mimics, and the opposite result was observed after inhibiting miR-3165-5p (Fig. 2F, G). These results signified that miR-3156-5p enhanced the proliferation of HCC cells and angiogenesis of endothelial cells.

3.3 miR-3156-5p targeted SOCS5

We predicted the target genes of miR-3156-5p via online websites. The analysis showed that miR-3156-5p targeted the 3'UTR of SOCS5 (Fig. 3A). Then, a dual-luciferase reporter assay was conducted to determine the relationship between the two. As a result, miR-3156-5p overexpression dramatically reduced the luciferase activity in HCC cells transfected with SOCS5 -3'-UTR-WT, but it had no effect on that of the mutant SOCS5 (Fig. 3B). Furthermore, we carried out a RIP experiment to ascertain the relationship between the two. It turned out that miR-3156-5p and SOCS5 were enriched by anti-Ago2 instead of anti-

IgG in HepG2 cells (Fig. 3C). We examined the expression of SOCS5 and its downstream molecules JAK2, STAT3 in HCC cells using qRT-PCR and WB and discovered that up-regulating miR-3156-5 declined the SOCS5 expression and heightened the phosphorylation of JAK2 and STAT3, while inhibiting miR-3156-5 had the opposite effect (Fig. 3D, E) These results testified that there was a negative binding relationship between miR-3156-5p and SOCS5.

3.4 Overexpressing SOCS5 dampened the proliferation of HCC cells and the tube formation of endothelial cells under hypoxia

To understand the impact of SOCS5 on HepG2 cells and HUVECs, we transfected Vectors, SOCS5 overexpression plasmids, and SOCS5 overexpression plasmids + miR-3156-5p mimics into HCC cells. qRT-PCR results manifested that HepG2 cells transfected with SOCS5 overexpression plasmids exhibited high expression of SOCS5 (vs. the Vector group). However, the SOCS5 profile was choked after up-regulating miR-3156-5p (Fig. 4A). WB results illustrated that SOCS5 was up-regulated and the phosphorylation of JAK2 and STAT3 was hampered after up-regulating SOCS5 (vs. the Vector group). On the contrary, up-regulating miR-3156-5p led to the opposite result (Fig. 4B). CCK-8 and Brdu experiments revealed that SOCS5 evidently restrained HepG2 cell proliferation (vs. the Vector group), while cell proliferation was heightened after up-regulating miR-3156-5p (Fig. 4C, D). In addition, we checked the expression of HIF-1α and VEGFA in HepG2 cells with qRT-PCR and WB. As a result, the profiles of HIF-1α and VEGFA were hampered after SOCS5 up-regulation (vs. the Vector group). Compared with the SOCS5 group, the expression of HIF-1α and VEGFA in HepG2 cells was elevated after up-regulating miR-3156-5p (Fig. 4E, F). These results hinted that overexpressing SOCS5 abated HCC cell proliferation.

3.5 Overexpressing miR-3156-5p boosted angiogenesis and tumor growth *in vivo*

HepG2 cells transfected with miR-3156-5p mimics and miR-NC were subcutaneously injected into both armpits of female BALB/c nude mice. Compared with the miR-NC group, mice in the miR-3156-5p group had larger volume and more weight (Fig. 5A-C). IHC was adopted to assess the profiles of SOCS5, STAT3, HIF-1α and VEGF in tumor tissues of mice. It turned out that the SOCS5 expression in the HepG2-miR-3156-5p group was lower, while the levels of STAT3, HIF-1α and VEGFA were higher than those in the HepG2 group (Fig. 6D-G). In summary, miR-3156-5p abated tumor growth and angiogenesis *in vivo* by curbing SOCS5 and up-regulating the HIF1α/VEGFA axis.

4. Discussion

HCC is the most frequent liver cancer and the primary cause of cancer-related death worldwide [23]. Increasing evidence manifests that hypoxia, which is typical during cancer, regulates cancer cell metabolism [24]. The oxygen-sensitive transcriptional activator 1 (HIF-1) is a key transcriptional mediator in response to hypoxia. HIF-1 seems to be involved in the formation of the vascular system through synergistic associations with other pro-angiogenic factors, such as VEGF, placental growth factor (PlGF)

or angiogenin during the physiological or pathological process [25]. Angiogenesis is a cancer marker, and endothelial cell proliferation, migration and activation are related to tumor angiogenesis [26]. This study demonstrates that miR-3156-5p may activate the HIF-1α/VEGF pathway by abating SOCS5, thus enhancing the angiogenic potential of endothelial cells under hypoxia. The miR-3156-5p/SOCS5/HIF-1α/VEGF axis is an underlying novel molecular target for the diagnosis and treatment of HCC.

Studies have reported that dysregulation of miRNAs is associated with various malignancies in humans [27]. Interestingly, miRNAs can target various different downstream effectors in different tumors. Due to the complexity of the downstream regulatory network of miRNAs and their tissue specificity, some target genes accelerate tumor progression while others exert anti-tumor effects [28, 29]. Therefore, miRNAs in different tumors may have different functions. In hypoxia-induced HCC, miR-199a-3p [30], miR-512-3p [31], miR-3677-3p [32], and miR-140 [33] have been found to be aberrantly expressed. Although miR-3156-5p's expression and role in HCC have not been investigated, it is highly expressed in breast cancer tissues [11] and colorectal cancer tissues [34] and is associated with malignant tumor development. Here, we discovered that miR-3156-5p was up-regulated in HCC cell lines under hypoxia, and up-regulated miR-3156-5p facilitated HCC cell proliferation and the tube formation of endothelial cells by elevating HIF-1α/VEGFA expression. In contrast, down-regulating miR-3156-5p exerted the reverse effect. These findings confirmed that miR-3156-5p contributed to HCC.

SOCS5 is a negative regulator of the JAK-STAT pathway and is often down-regulated in various cancers [35–37]. SOCS5 activates the JAK/STAT signaling in HCC, thereby enhancing cancer stemness and chemoresistance [38]. Up-regulating SOCS5 suppresses the JAK-STAT3 pathway to abate the proliferation, migration and invasion of HCC cells [39]. These researches demonstrate that the low expression of SOCS5 is associated with the malignant development of HCC. Besides, SOCS5 is a key endogenous feedback inhibitor of pathological angiogenesis [40]. miR-301a lowers SOCS5 expression, activates JAK/STAT3 and increases angiogenesis in endothelial cells in pancreatic cancer [41]. Also, miR-141-3p reduces the SOCS5 expression, leading to the up-regulation of the JAK-STAT3 pathway in endothelial cells and heightening endothelial angiogenesis in epithelial ovarian cancer [42]. Based on the above studies, we explored the role of SOCS5 in cell proliferation and angiogenesis in HCC. We found that SOCS5 was down-regulated in hypoxia-induced HCC cells, and overexpressing SOCS5 reduced HCC cells' proliferation and endothelial cells' tube formation. Meanwhile, overexpressing miR-3156-5p weakened the effect of SOCS5. The target gene of miR-3156-5p was predicted using an online website, and miR-3156-5p was found to be one. What's more, the negative binding correlation between the two was ascertained with the dual-luciferase reporter assay and RIP assay. These results uncovered that miR-3156-5p targeted and regulated SOCS5 to affect the proliferation of HCC cells and vascular formation of endothelial cells.

HIF-1 is an oxygen-dependent transcriptional activator, and its expression in HCC tissues is related to tumor differentiation and intrahepatic and extrahepatic metastasis [43–46]. HIF-1α is a unique O_2 regulatory subunit that determines the activity of HIF-1. It elevates a series of genes and proteins related to tumor cell survival rate under hypoxia, including VEGF, insulin-like growth factor, inducible nitric oxide synthase, and platelet-derived endothelial growth factor, thereby leading to angiogenesis and massive

tumor growth. The HIF-1α/VEGFA pathway is the most studied regarding the regulation of hypoxia/ischemia-induced angiogenesis [45]. Asparagus polysaccharide (ASP) partially restrains HCC cell migration, invasion, and angiogenesis by impeding the HIF-1α/VEGF pathway [46]. Sulforaphane (SFN) abates HCC cell-induced angiogenesis by attenuating the STAT3/HIF-1α/VEGF signal transduction [47]. The mechanism of drugs and HIF-1α/VEGF in HCC has been extensively studied, but the specific mechanism of miR-3156-5p in HCC cell proliferation and angiogenesis remains unclear. Therefore, we conducted the current study and discovered that HIF-1α/VEGF was highly expressed in hypoxia-induced HCC cells. Meanwhile, miR-3156-5p is positively correlated with HIF-1α/VEGFA, and overexpressing miR-3156-5p increases HIF-1α/VEGFA expression. These results testify that the oncogenic effect of miR-3156-5p is mediated, at least in part, by accelerating the HIF-1α/VEGFA expression.

5. Conclusions

Overall, miR-3156-5p up-regulates HIF-1α/VEGFA by dampening SOCS5, thus partially weakening HCC cell proliferation and angiogenesis. These findings help formulate the treatment plan for HCC and provide necessary help for the prognosis of HCC. However, our study had several limitations. First, our results need to be validated in other HCC cell lines, such as Huh7, bel7404, and SMMC-7721. Second, our experiments need to be verified in a larger number of *in-vivo* experiments.

Abbreviations

miRNAs
MicroRNAs; HCC:hepatocellular carcinoma; CM:conditioned medium
qRT-PCR
Quantitative reverse transcription PCR; CCK-8:cell counting kit-8
HBV
hepatitis B virus; HCV:hepatitis C virus; SOCS:suppressor of cytokine signaling;
TAE/TACE
Transcranial arterial embolization or chemical embolization;
VEGF
vascular endothelial growth factor; FGFRL1:fibroblast growth factor receptor 1;
HIF-1α
hypoxic-inducible factor-1α; HUVECs:Human umbilical vein endothelial cells;
BrdU
Bromo-2-deoxyUridine; WB:Western blot; RIP:RNA immunoprecipitation;
PlGF
placental growth factor; ASP:Asparagus polysaccharide; SNF:Sulforaphane.

Declarations

Ethics approval and consent to participate

Our study was approved by the Ethics Committee of the First Hospital of Lanzhou University and were in line with the Guidelines of National Institutes of Health on animal care and use.

Consent for publication

Not applicable.

Availability of data and materials

The data sets used and analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

Conceived and designed the experiments: Zheng Guo;

Performed the experiments: Bin Tie, Zheng Guo;

Statistical analysis: Li Li, Wenhui Wang, Rong Liu, Jin Fang;

Wrote the paper: Bin Tie.

All authors read and approved the final manuscript.

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Figures

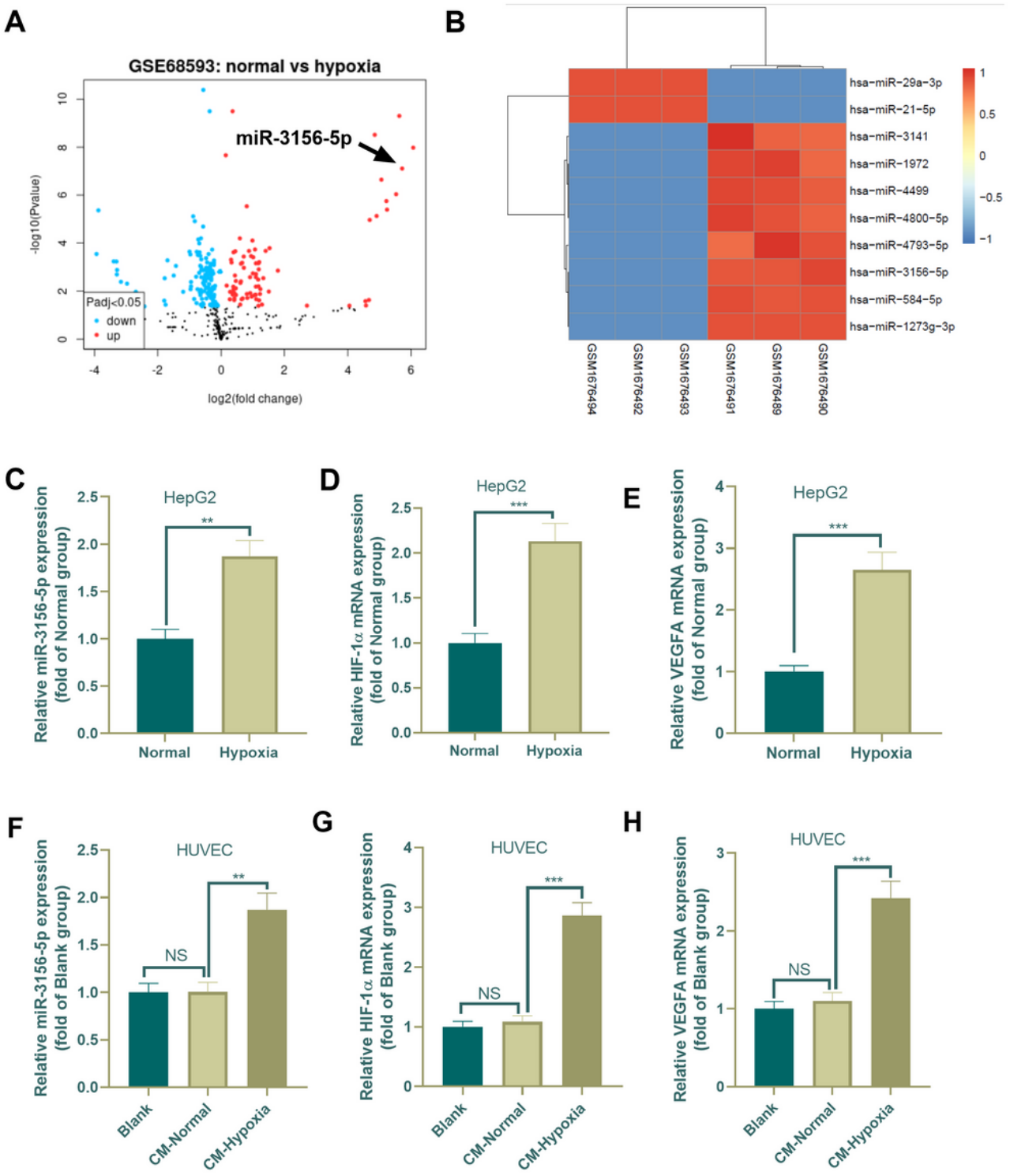

Figure 1

miR-3156-5p, HIF-1α and VEGFA were up-regulated in HCC cells and endothelial cells under The microarray data was submitted to GEO database and the GEO accession number was GSE68593. A.The volcanic plot revealed that miR-3156-5p was overexpressed in HCC under hypoxic condition. B. The heat map uncovered the significantly highly or lowly expression genes in HCC cells and hypoxia-induced HCC cells. C-E. The miR-3156-5p, HIF-1α and VEGFA expression in HepG2 cells was examined by qRT-PCR under normal or hypoxic conditions. **P<0.01, ***P<0.001(vs. Normoxia group). F-H: The miR-3156-5p, HIF-1α and VEGFA levels in HUVECs co-cultured with HepG2 were compared by qRT-PCR. NSP > 0.01 (vs Blank group), ** P < 0.01, ***P < 0.001 (vs Normoxia group). Data were presented as mean ±SD (n =3).

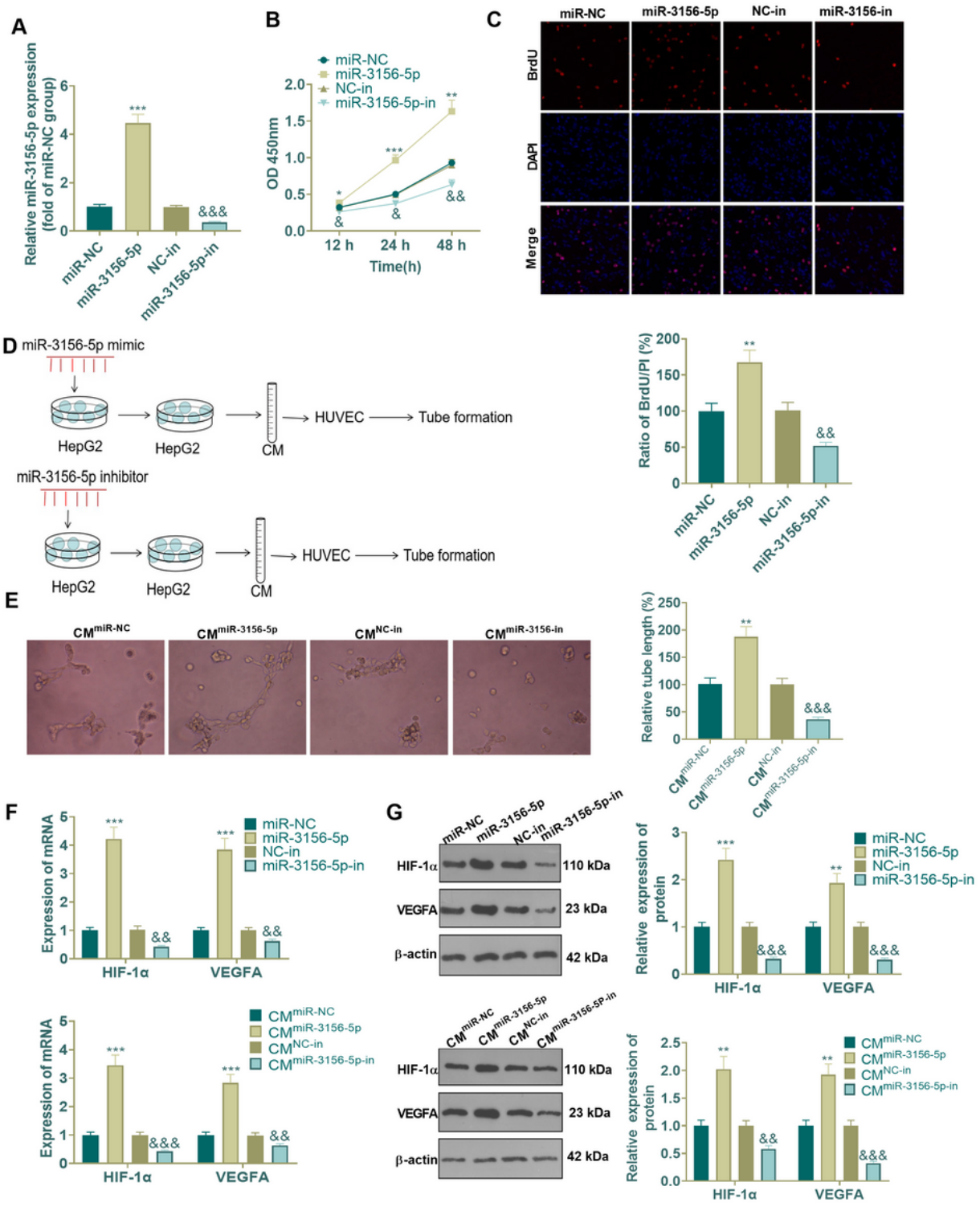

Figure 2

miR-3156-5p facilitated the proliferation of HCC cells and tube formation of endothelial cells A: The miR-3156-5p profile in HepG2 cells after transfection with miR-3156-5p mimics and inhibitors was determined by qRT-PCR. B and C: The proliferation of HepG2 cells transfected with miR-3156-5p mimics and inhibitors was monitored by the CCK-8 assay and BrdU staining. *P <0.05, **P <0.01, ***P <0.001 (vs.miR-NC group), && P <0.01, &&& P <0.001 (vs.NC-in group). D Schematic diagram of sample handling,

collection, and testing. HCC cells were transfected with miR-3156-5p mimics (50 nM) and miR-3156-5p inhibitors (50 nM) for 48 hours. CM was harvested for culturing primary HUVECs. E: The tube formation test was applied to verify the tube formation of HUVECs. A representative picture of tube formation was shown on the left, and a statistical graph of the length of small tubes was exhibited on the right. *** P<0.001 (vs.CMmiR-NC group), &&& P <0.001 (vs.CMNC-in group). F and G: The mRNA and protein expression of HIF-1α and VEGFA in HepG2 cells and HUVECs were tested by qRT-PCR and WB. ** P <0.01, *** P <0.001 (vs.miR-NC group,CMmiR-NC group). && P <0.01, && P <0.001 (vs. NC-in group, CMNC-IN group). Data were expressed as mean ±SD (n =3).

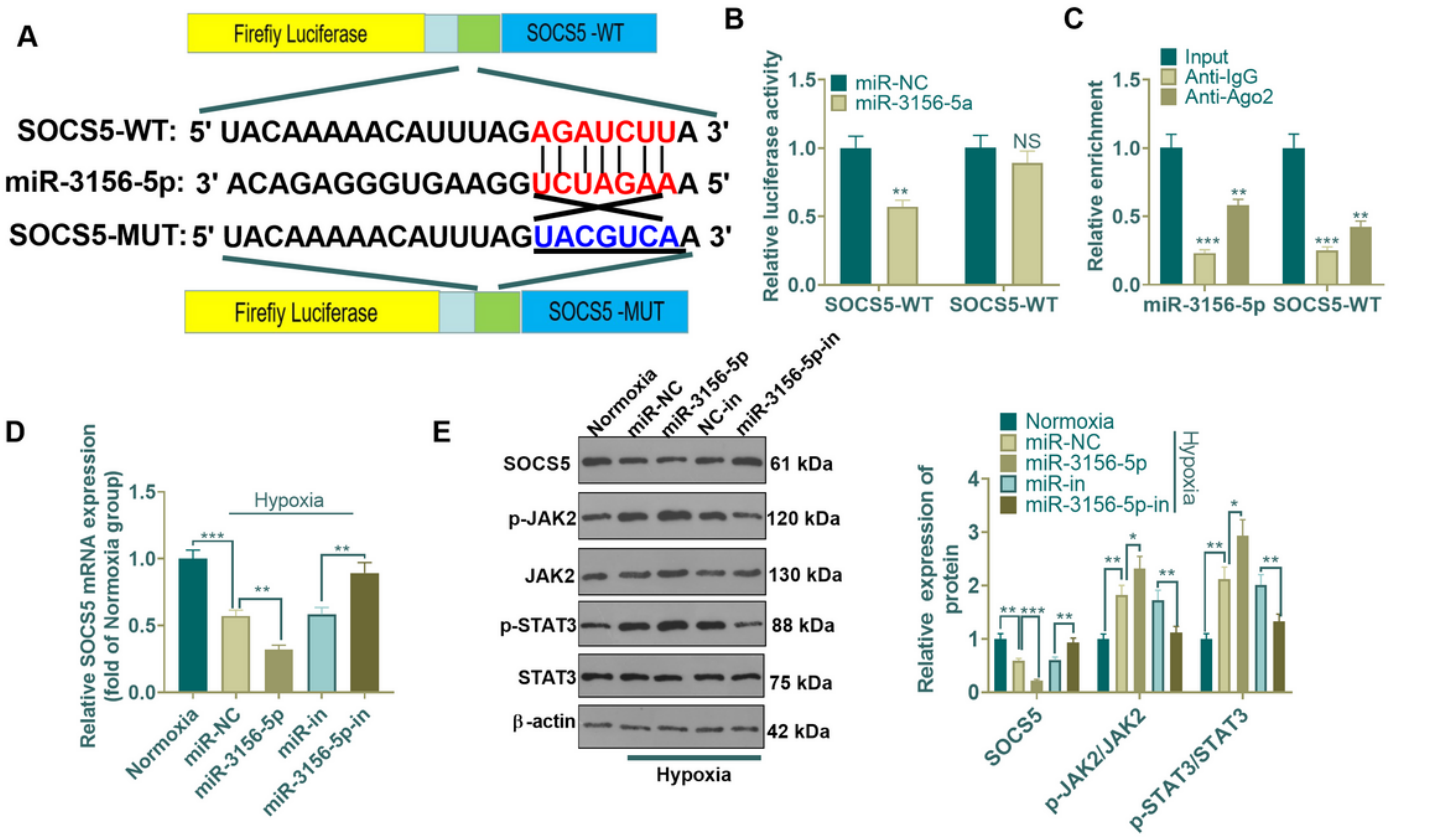

Figure 3

miR-3156-5p targeted SOCS5 A: The online database predicted binding sites between SOCS5 and miR-3156-5p. B: The predicted binding sequence of SOCS5 in miR-3156-5p was verified by the dual-luciferase reporter assay under hypoxia. C: RIP analysis showed that there was a direct correlation between miR-3156-5p and SOCS5 in HepG2 cells under hypoxia. NSP>0.01, **P<0.01, ***P<0.001(vs miR-NC group). D: SOCS5 expression in HepG2 cells after transfection of miR-3156-5p mimics and miR-3156-5p inhibitors under hypoxia. ***P < 0.001 (vs Normoxia group), *P<0.05, **P<0.01, ***P<0.01 (vs.miR-NC, vs.NC-in group). Data were represented as mean ±SD (n =3).

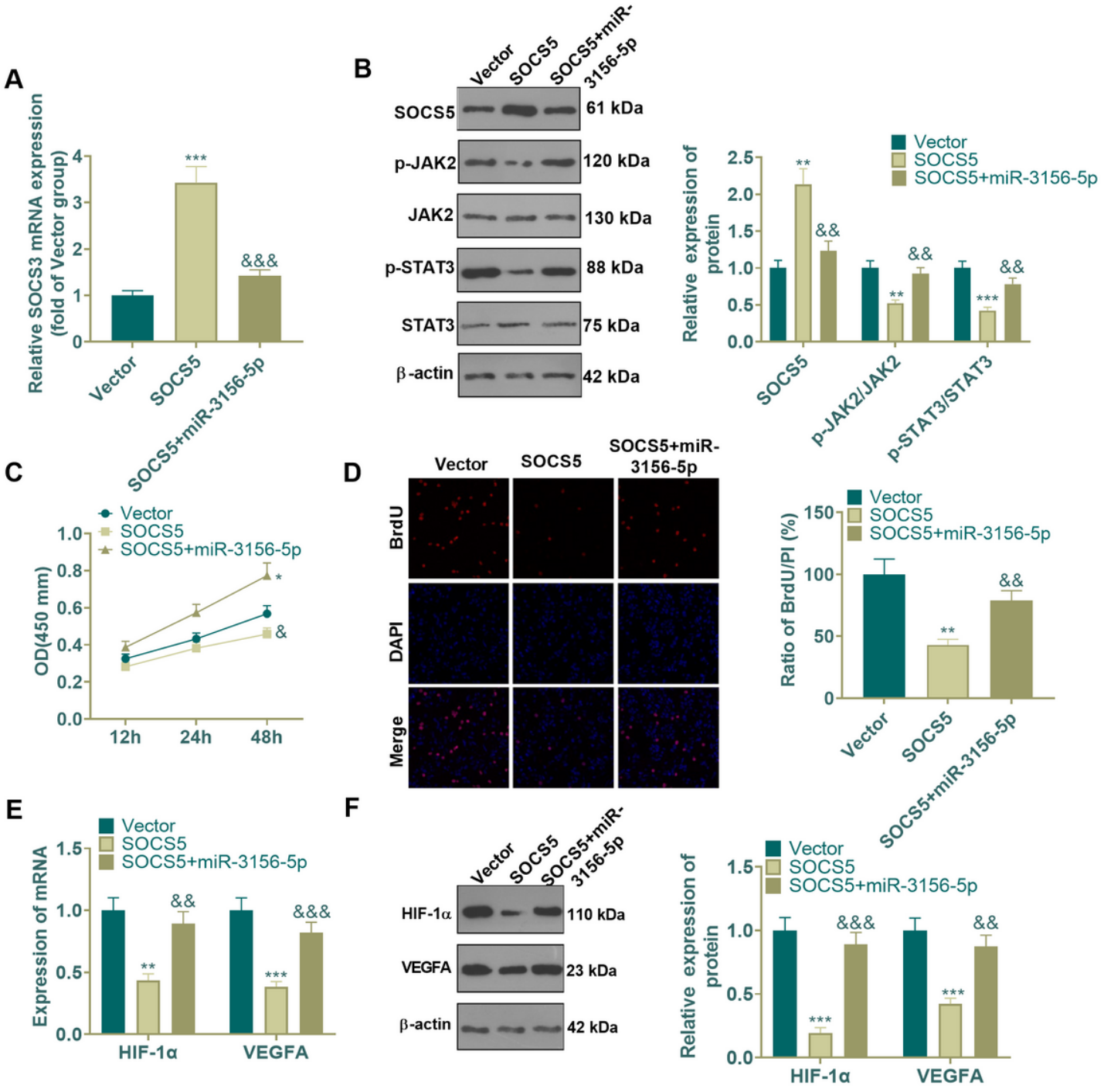

Figure 4

Overexpressing SOCS5 reduced hypoxia-induced HCC cell proliferation and endothelial cell tube formation Vectors, SOCS5 overexpression plasmids, and SOCS5 overexpression plasmids + miR-3156-5p mimics were transfected into HCC cells. A: qRT-PCR was implemented to examine the SOCS5 expression in HepG2 cells. B The expression of SOCS5, JAK2 and STAT3 in HepG2 cells was monitored by WB. C and D: HepG2 cell proliferation was checked by CCK-8 and BrdU assay. E and F: The mRNA and protein expression of HIF-1α and VEGFA in HepG2 cells was monitored by qRT-PCR and WB. * P <0.05, ** P <0.01,

*** P <0.001 (vs.Vector group), & P <0.05, && P <0.01, &&& P <0.001 (vs.SOCS5 group). Data were expressed as mean ±SD (n =3).

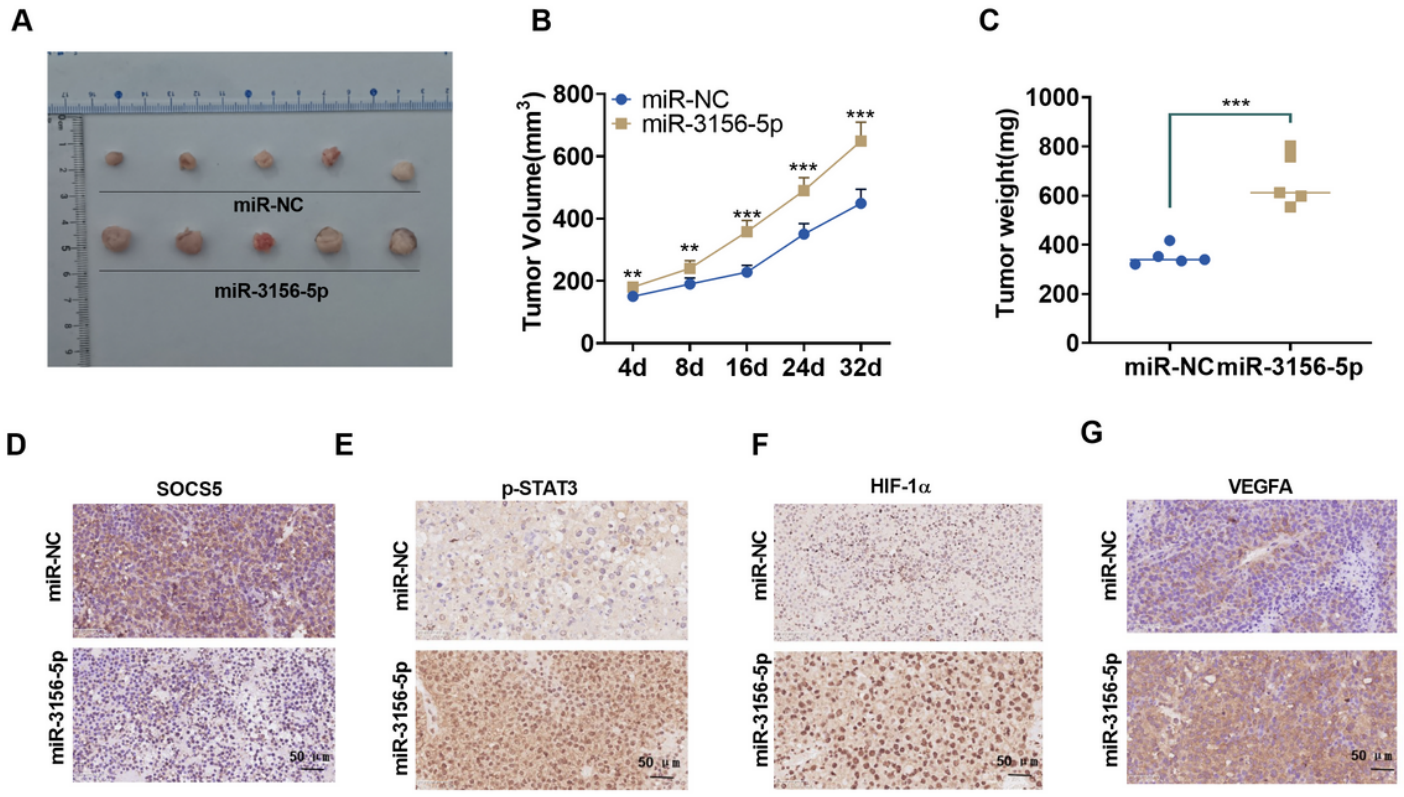

Figure 5

Overexpression of miR-3156-5p down-regulated SOCS5 to regulate the HIF-1α/VEGF pathway and increase angiogenesis and tumor growth in vivo A and B: Volume of xenograft tumors injected with miR-NC and miR-3156-5p. C: Weight of xenograft tumors injected with miR-NC and miR-3156-5p. C: IHC was conducted to examine the expression of SOCS5, HIF-1α, and VEGF in tumor tissues. *P<0.05, ***P<0.001 (vs. miR-NC group). Data were presented as mean ±SD (n =5).

Supplementary Files

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