miR-451 acts as a suppressor of angiogenesis in hepatocellular carcinoma by targeting the IL-6R-STAT3 pathway

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Abstract. Hepatocellular carcinoma (HCC) is a highly vascularized tumor and the third ranking contributor of tumor-associated death. Our previous study corroborated the inhibitory roles of miRNA-451 (miR-451) in HCC cell growth and invasion. However, its effect on angiogenesis in HCC remains poorly elucidated. In this study, overexpression of miR-451 clearly attenuated the promoting effects of HCC cells on cell proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs). Importantly, ectopic expression of miR-451 also attenuated tumor growth and angiogenesis in nude mice. *In vitro*, the expression of IL-6 receptor (IL-6R) was reduced and identified as a direct target of miR-451 by bioinformatics and a dual-firefly luciferase reporter assay. Moreover, upregulation of IL-6R strikingly ameliorated the inhibitory function of conditioned medium from miR-451-transfected HCC cells in HUVEC proliferation, migration and tube formation. Further mechanistic assay substantiated that miR-451 restrained vascular endothelial growth factor (VEGF) production of HCC cells by targeting IL-6R-STAT3 signaling as evidenced that IL-6R upregulation induced the increase in VEGF levels and interrupting signal transducer and activator of transcription 3 (STAT3) signaling with ectopic expression of dominant-negative STAT3 (STAT3D) markedly decreased VEGF expression. Additionally, conditioned medium of miR-451-overexpressed HCC also impaired the VEGF receptor 2 (VEGFR2) signaling in HUVECs. Accordingly, miR-451 may function as a potential suppressor of tumor angiogenesis in HCC by targeting IL-6R-STAT3-VEGF signaling, suggesting a promising therapeutic avenue for managing HCC.

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Introduction

Hepatocellular carcinoma (HCC), a common aggressive carcinoma of the liver, ranks as the third contributor for tumor-associated death around the world (1,2). Despite recent advances, there is still an annual incidence of >560,000 deaths and a dismal 10% five-year overall survival rate (2,3). The precise molecular mechanisms underlying the pathological progression of HCC remain poorly elucidated.

During the past few years, increasing evidence has identified HCC as a highly vascularized tumor with high invasion and metastasis, which contributes to tumor recurrence and poor survival of HCC patients (4,5). It is widely accepted that angiogenesis is a prerequisite for the development and metastasis of carcinoma by supplying nutrients and oxygen (6,7). A great number of factors have been reported to be involved in tumor angiogenesis, especially the vascular endothelial growth factor (VEGF) (8,9). Targeting cancer vasculature to 'starve a tumor to death' has become a new approach for carcinoma therapy (7,10). Though many anti-angiogenic drugs have been developed to investigate their effect on human malignancies, the efficacy is modest (11). Thus, it is urgent to develop more effective therapy against HCC.

MicroRNAs (miRNAs) are evolutionarily conserved non-coding RNAs with 22-nucleotide length and can act as the negative regulators of target genes by interacting with the 3'-untranslated region (3'UTR). miRNAs have been corroborated to be associated with a variety of biological processes, including cell proliferation, invasion, angiogenesis and fat metabolism (12-15). Recently, emerging evidence has confirmed the deregulated expression of miRNAs in carcinomas, including HCC (16,17). Among them, miRNA-451 (miR-451) has drawn increasing interest due to its prominent function in the development of some cancers, such as HCC (16). In previous research reports the decrease of miR-451 in gastric cancer tissues and its downregulation tends to be positively correlated with lymphatic metastasis and overall survival of patients (17). A remarkable reduction of miR-451 has been validated in HCC cells (16). Additionally, its elevation obviously delay cell growth and invasion in HCC. However, no report exists addressing its roles in angiogenesis of HCC.

In the present study, we aimed to investigate the effects of miR-451 expression on angiogenesis in HCC. Moreover, the underlying mechanism was explored.

Materials and methods

Antibodies and reagents. Rabbit polyclonal antibodies to IL-6 receptor (IL-6R) and proliferating cell nuclear antigen (PCNA) were acquired from Abcam (Cambridge, UK). Antibodies against signal transducer and activator of transcription 3 (STAT3) and phosphorylated STAT3 (p-STAT3-Tyr705) were obtained from Cell Signaling Technology. Rabbit polyclonal antibodies against VEGF receptor 2 (VEGFR2) and phospho-Tyr1175-VEGFR2 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). The anti-VEGF antibody was from (R&D Systems, Minneapolis, MN, USA). Antibodies against ERK1/2 and phospho-T202/Y204-ERK1/2 were from BD Biosciences (Franklin Lakes, NJ, USA).

Cell culture. The human hepatoma cell lines HepG2 and HEK293T were purchased from American Type Culture Collection (ATCC; Rockville, MD, USA). The umbilical vein endothelial cells (HUVECs) were obtained from AllCells (Shanghai, China). HepG2 and HEK293T cells were incubated with Dulbecco's modified Eagle's medium (DMEM) (Invitrogen Corp., Grand Island, NY, USA) containing 10% fetal bovine serum. HUVECs were cultured in RPMI-1640 medium (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin and 100 g/ml streptomycin. All cells were incubated in a humidified atmosphere at 37˚C with 5% CO_2.

Oligonucleotide transfection. Lentivirus-based plasmids for constitutive expression of miR-451 or scrambled miRNA (miR-con, used as NC) and the virus packaging kit were obtained from GeneCopoeia (Rockville, MD, USA). Following co-transfection into HEK293T using EndoFectin Lenti transfection reagent according to the manufacturer's instructions About 48 h later, virus titers were collected and evaluated by the p24 ELISA kit (Cell Biolabs, Inc., San Diego, CA, USA). Then, the obtained lentiviral particles were transduced into HepG2 cells for 24 h. The stable miR-451-overexpressing cells were selected using puromycin for additional 3 days.

Collection of the tumor-conditioned medium (TCM). HepG2 cells with miR-451 were preconditioning with pcDNA-IL-6R lacking 3'UTR, pIRES-STAT3β [a dominant-negative STAT3 (STAT3D)] or vehicle (GeneChem, Shanghai, China). About 12 h later, cells were incubated in DMEM medium for further 14 h. Then, the TCM was centrifuged sequentially at 500 g to discard the detached cells, followed by 12,000 g centrifugation to remove cell debris at 4˚C for 15 min. The supernatant was then gathered and stored at -80˚C for the subsequent experiments.

Luciferase reporter assay. The wild-type (wt) and mutant (mut) 3'UTR of IL-6R predicted to interact with miR-451 were constructed and cloned to the firefly luciferase-expressing vector psiCHECK™ (Promega, Madison, WI, USA). HEK293T cells were seeded into a 96-well plate and co-transfected with wt-IL-6R or mut-IL-6R 3'UTR reporter vector, 5 ng of pRL-TK, and miR-451 or miR-con with the help of Lipofectamine 2000 reagent (Invitrogen Corp., Carlsbad, CA,

USA). After 48 h incubation, luciferase activities were detected by Dual-Luciferase Reporter system (Promega).

RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR). After treatment under various conditions, the HepG2 cells were lysed with TRI reagent (Sigma) to extract total RNA. Then, the reverse transcription was performed to synthesize the cDNA using a High Capacity cDNA Archive kit (Applied Biosystems, Foster City, CA, USA). The subsequent qRT-PCR was carried out to evaluate the relative expression of mRNA using the miScript SYBR®-Green PCR kit (Qiagen, China) and SYBR-Green I (Molecular Probes, Invitrogen Corp.) for miR-451 and other molecules. All reaction conditions and processes were implemented according to the manufacturer's instructions. The specific primers for miR-451, IL-6R and VEGF were used as previously published (8,18). The expression levels were normalized using U6 for miR-451 and β-actin for other genes. All data were analyzed using the $2^{-\Delta\Delta Ct}$ equation.

Western blotting. Cells were solubilized in lysing buffer (Beyotime, Nantong, China) and the extracted protein concentration was measured using the BCA assay (Pierce, Rockford, IL, USA). Then, about 40 μg of proteins was subjected to 12% SDS-PAGE, followed by the transfer to PVDF membrane (Millipore, Bedford, MA, USA). After blocking with 5% non-fat milk, the membrane was probed with primary antibodies against human IL-6R, PCNA, VEGF, STAT3, p-STAT3, VEGFR2, p-VEGFR2, ERK and p-ERK. Then, HRP-conjugated secondary antibodies were added for further incubation of 1 h. To visualize the bound antibodies, the LumiGLO reagent (Pierce) was introduced. The β-actin was used as protein loading control. All band intensities were quantified using a Gel Doc™ XR imaging system and Quantity One (Bio-Rad, USA).

Cell viability assay. Cell viability was monitored by Cell Counting kit (CCK)-8 (Dojindo, Kumamoto, Japan). Briefly, HUVECs were seeded onto 96-well plates at the density of $5x10^3$ cells/well. Then, cells were incubated with the TCM collected from different background of miR-451 expression for the indicated times (12, 24 and 36 h). Subsequently, 10 μl of CCK8 reagents were added for further 2 h incubation at 37˚C. The absorbance at 450 nm was measured to assess the number of viable cells by a Safire 2 microplate reader (Tecan, Switzerland). Relative cell viability was shown as the absorbance percentage of the treatment group to the control group.

In vitro migration assay. Cell migration was evaluated using the scratch wound assay. After seeding in 24-well plates, HUVECs were cultured with various TCM. Then, a single scratch wound was formed by scraping the cell layer using a tip of 200 μl pipette. About 24 h later, the scratch wounds were visualized by an inverted microscope. The scratch wound width was quantified to assess cell migration ability of HUVECs using the ImageJ software.

Tube formation analysis in vitro. The 96-well culture plates were precoated with Matrigel (BD Pharmingen, San Jose, CA, USA) overnight. Then, HUVECs were seeded into the plate
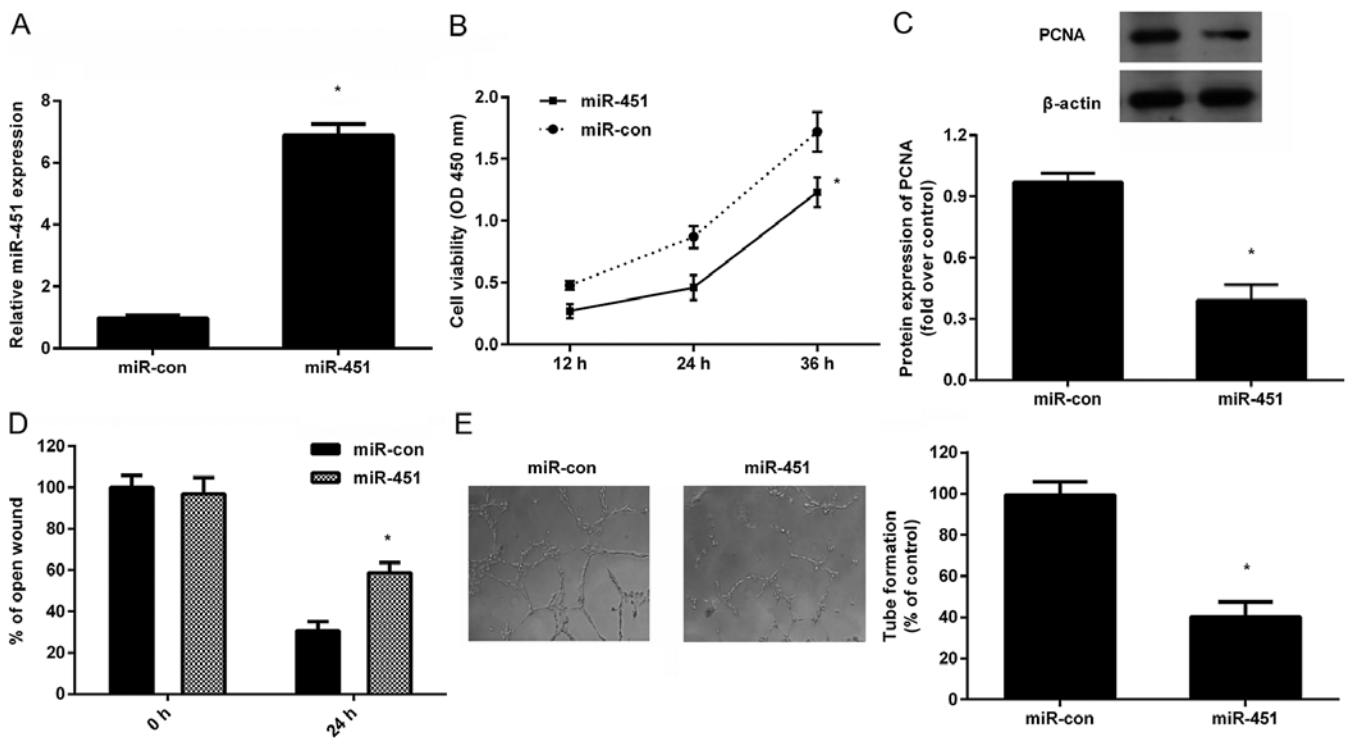
Figure 1. Upregulation of miRNA-451 (miR-451) in HepG2 cells mitigated cell proliferation, migration and tube formation of human umbilical vein endothelial cells (HUVECs). (A) Following transfection with miR-451, the transfection effects were evaluated by quantitative real-time polymerase chain reaction (qRT-PCR). After incubation with tumor-conditioned medium (TCM) from HepG2 cells transfected with miR-451 or miR-con, (B) the function in cell proliferation, (C) proliferating cell nuclear antigen (PCNA) expression, (D) migration and (E) capillary tube formation were measured. *P<0.05 vs. miR-con groups.

at the density of $1x10^4$ cells/well and cultured in the absence or presence of various TCM from HepG2 cells. The formation of capillary-like structures were then analyzed at 24-post incubation and photographed under an inverted microscope. Tube formation was evaluated by counting branch points in five random fields per well.

Xenograft model of HCC in nude mice. For xenograft implantation experiments, male BALB/c nude mice aged 4-6 weeks were used and obtained from the Hunan Slac Jingda Laboratory Animal Co., Ltd. (Changsha, China). All animals were housed under specific pathogen-free conditions and used according to the guidelines of the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Animal care and procedures were approved by the Institutional Animal Care and Use Committee of the First Affiliated Hospital of Xi'an Jiaotong University. HepG2 cells ($5x10^6$) transfected with miR-451 or control were subcutaneously injected into mice to establish xenograft models. Tumor size was detected every 4 days and the tumor volumes (10 animals/group) were calculated with the following formula: Tumor volume = (largest diameter x perpendicular height^2)/2. Five weeks later, the animals were euthanized using sodium pentobarbital and tumors were removed.

Angiogenesis assay in vivo. Tumors from mice were collected and fixed in formalin. Then, the specimens were processed for paraffin embedding and subsequently cut into standard 6-μm sections. To evaluate angiogenesis, immunofluorescence was performed. After blocking endogenous peroxidase

activity and non-specific bind, the primary antibodies against CD31 (eBioscience) were added. Then, the samples were incubated with biotin-linked donkey anti-rat and Texas Red Streptavidin (Jackson ImmunoResearch, West Grove, PA, USA), followed by the counterstain with DAPI (Sigma). The specimens were photographed under a Zeiss LSM 510 confocal microscope and blood vessel areas were calculated by the following formula: % Area = total red signal/total DAPI signal.

ELISA. The serum from the mice and TCM were collected. Then, the equal volume of samples was subjected to ELISA to determine the VEGF concentration using a commercial VEGF ELISA kit (R&D Systems). All procedures were performed according to the manufacturer's instructions.

Statistical analysis. Data were analyzed by SPSS 11.0 and shown as means ± standard deviation (SD). All experiments were performed at least three times. Comparisons among different groups were analyzed based on Student's t-test and ANOVA. P<0.05 was considered as statistically significant.

Results

miR-451 evidently antagonizes proliferation, migration and tube formation of HUVECs. To investigate the biological significance of miR-451 in HCC angiogenesis, its effect on cell proliferation, migration and tube formation of HUVECs were explored. As shown in Fig. 1A, a pronounced increase of miR-451 was validated in HepG2 cells after transfection
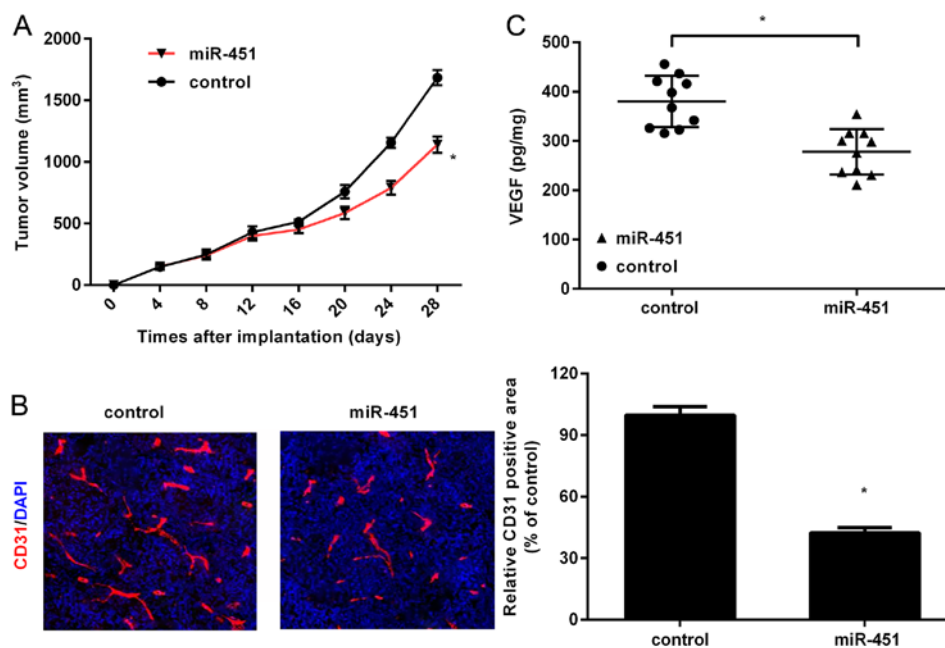
Figure 2. Effect of miRNA-451 (miR-451) on angiogenesis of hepatocellular carcinoma (HCC) *in vivo*. HepG2 cells transfected with miR-451, or control, were subcutaneously injected into BALB/c nude mice. (A) The effect on tumor growth was detected. (B) Five weeks later, the animals were euthanized and tumor tissues were stained for CD31 by immunofluorescence to evaluate blood vessel densities. (C) The serum levels of vascular endothelial growth factor (VEGF) were determined using ELISA kit. *P<0.05.

with miR-451. Further analysis demonstrated that incubation with TCM from miR-451-overexpressed HepG2 cells strikingly decreased HUVEC proliferation in a time-dependent manner (Fig. 1B). Consistently, the expression of PCNA, a marker for cell proliferation, was also downregulated in HUVECs when treated with the TCM (Fig. 1C). Moreover, TCM from miR-451-transfected HepG2 cells notably mitigated cell recruitment of HUVECs (Fig. 1D). Importantly, a remarkable inhibition in capillary tube formation of HUVECs was substantiated when HUVECs were grown in TCM obtained from miR-451-elevated HepG2 cells. Accordingly, these data suggested that miR-451 upregulation in HCC cells might inhibit angiogenesis of HUVECs *in vitro*.

Ectopic expression of miR-451 mitigates tumor growth and angiogenesis in vivo. To further elucidate the function of miR-451 on the development of carcinoma, HepG2 cells stably expressing miR-451 or control were subcutaneously injected into BALB/c nude mice. Interestingly, a noticeable decrease in tumor volume was observed in HepG2-miR-451 tumors in contrast to control groups (Fig. 2A), indicating that miR-451 could suppress tumor growth *in vivo*. The obvious decrease in blood vessel density was corroborated in HepG2-miR-451 groups by detecting the levels of CD31 (Fig. 2B), a common marker for vascular formation. Concomitantly, ectopic expression of miR-451 in HepG2 tumors also triggered an analogous downregulation of VEGF concentration in serum in comparison with HepG2-control groups (Fig. 2C). These results indicated that miR-451 might act as a critical suppressor of angiogenesis in HCC.

IL-6R is a direct target of miR-451. Analysis was performed to predict the potential target of miR-451 using publicly available algorithms (TargetScan, PicTar and microRNA.org). Among

these genes, IL-6R was identified as a potential target based on a predicted binding site of miR-451 at its 3'UTR (Fig. 3A). It has been reported that IL-6/IL-6R signaling is involved in tumor angiogenesis (8). To clarify the underlying mechanism related to miR-451-mediated inhibitory effect on angiogenesis in tumors, the expression of IL-6R was assessed. As expected, overexpression of miR-451 markedly reduced the mRNA levels of IL-6R in HepG2 cells (Fig. 3B). Simultaneously, a similar decrease of IL-6R protein was also demonstrated following miR-451 transfection (Fig. 3C). Noticeably, the luciferase activity was markedly diminished following co-transfection of miR-451 with wt-IL-6R-3'UTR vector, but not in mut-IL-6R-3'UTR groups (Fig. 3D), indicating that IL-6R was a direct target of miR-451.

Overexpression of IL-6R attenuates the inhibitory effect of miR-451 on HUVEC proliferation, migration and tube formation. Based on the target relationship between miR-451 and IL-6R, we further explored whether miR-451 elicits its inhibitory role in angiogenesis of HUVECs by directly targeting IL-6R. Following transfection with pCDNA-IL-6R lacking 3'UTR, the inhibitory effect of TCM from miR-451-transfected HepG2 cells on HUVEC proliferation was obviously ameliorated (Fig. 3E). Consistently, the decreased migration of HUVECs triggered by miR-451 elevation was also attenuated in the above culture medium (Fig. 3F). Notably, a similar increase in capillary tube formation of HUVECs was also observed when HUVECs were incubated with TCM from HepG2 cells co-transfected with miR-451 and IL-6R. The above data confirmed that miR-451 could suppress angiogenesis of HUVECs *in vitro* by targeting IL-6R.

miR-451 suppresses VEGF production by blocking IL-6R-STAT3 signaling. VEGF is widely accepted as a
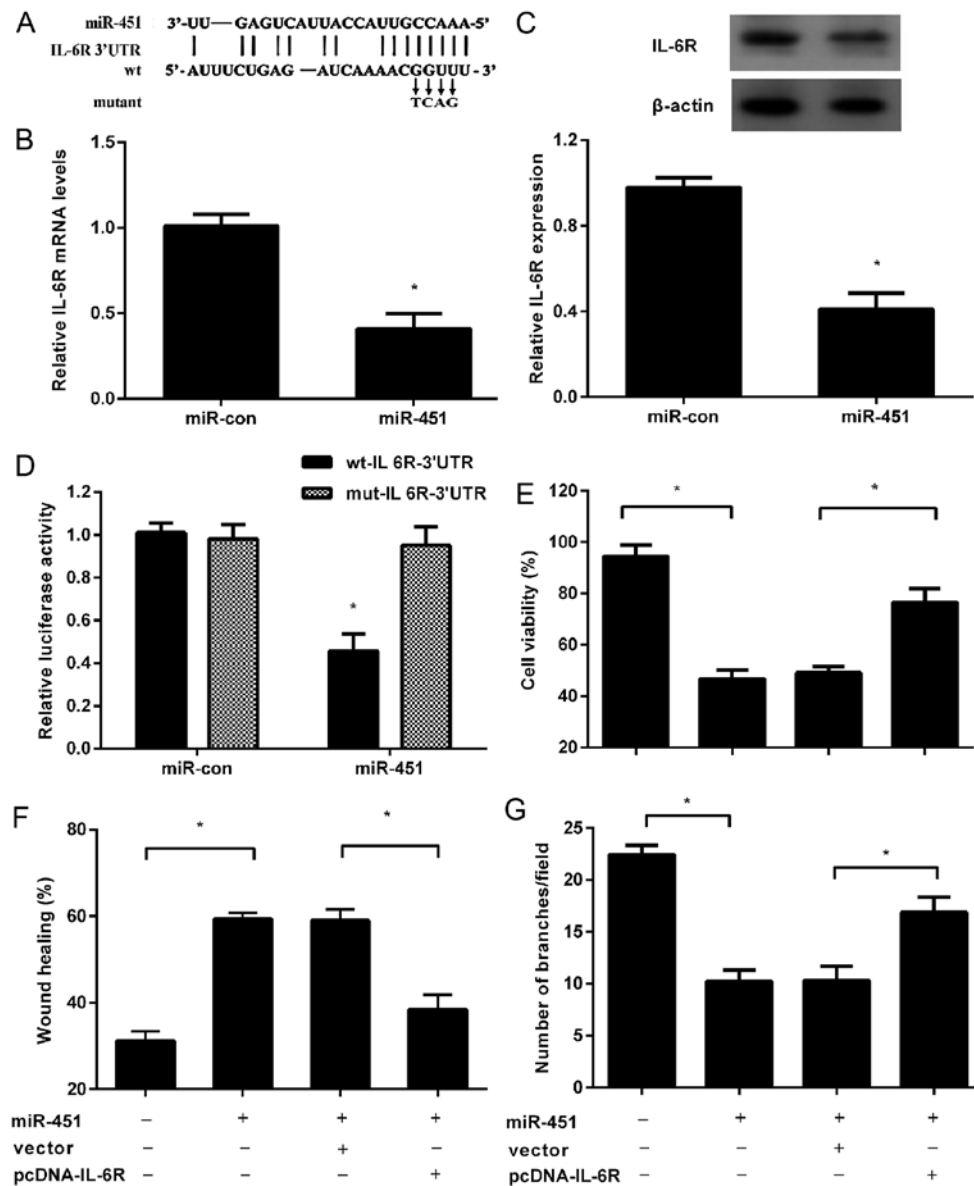
Figure 3. Elevation of miRNA-451 (miR-451) in HepG2 cells exerted anti-angiogenic effect on human umbilical vein endothelial cells (HUVECs) by targeting IL-6 receptor (IL-6R). (A) The putative binding sequences of miR-451 in the 3'-untranslated region (3'UTR) of IL-6R by bioinformatic analysis. HepG2 cells were transfected with miR-451 or miR-con. Then, (B) the microRNA (miRNA) and (C) the protein levels of IL-6R were analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) and western blotting. (D) Following transfection with reporter vectors containing wild-type (wt) or mutant (mut) IL-6R-3'UTR, the relative luciferase activity was assessed. Then, pcDNA-IL-6R lacking 3'UTR was transfected into HepG2 cells. The effects of tumor-conditioned medium (TCM) collected from the indicated groups on (E) cell viability, (F) cell migration and (G) tube formation were determined. *P<0.05.

vital regulator for angiogenesis. Accumulation evidence corroborates that IL-6/IL-6R exerts an important role in angiogenesis by activating STAT3-VEGF signaling (19,20). To further illustrate the underlying mechanism involved in miR-451-trigged inhibition on tumor angiogenesis, we investigated the expression of VEGF. Consistent with our hypothesis, elevation of miR-451 noticeably abrogated the mRNA level of VEGF (Fig. 4A). Moreover, the concentration of VEGF in conditioned medium of HepG2 cells was also reduced (Fig. 4B). Additionally, miR-451 upregulation significantly inhibited the STAT3 phosphorylation (Fig. 4C). Interestingly, IL-6R upregulation drastically antagonized the reduction of p-STAT3 and VEGF expression trigged by miR-451 overexpression (Fig. 4D). Furthermore, the increased expression of IL-6R obviously upregulated the concentration

of VEGF in TCM collected from miR-451-transfected HepG2 cells (Fig. 4E). Concomitantly, blocking STAT3 signaling with STAT3D noticeably suppressed VEGF expression (Fig. 4F) and concentration (Fig. 4G), implying that miR-451 could dampen VEGF production secreted by HCC cells through IL-6R-STAT3 signaling.

miR-451 inhibits VEGFR2 signaling in HUVECs. Convincing evidence indicates that tumor cell-produced VEGF can induce endothelial cell proliferation, migration and angiogenesis by activating VEGFR2, which then phosphorylates its down-stream ERK and subsequently induces angiogenesis (21). We further assess whether miR-451-decreased VEGF production by IL-6R-STAT3 signaling can abolish the VEGFR2 pathway in HEVECs.
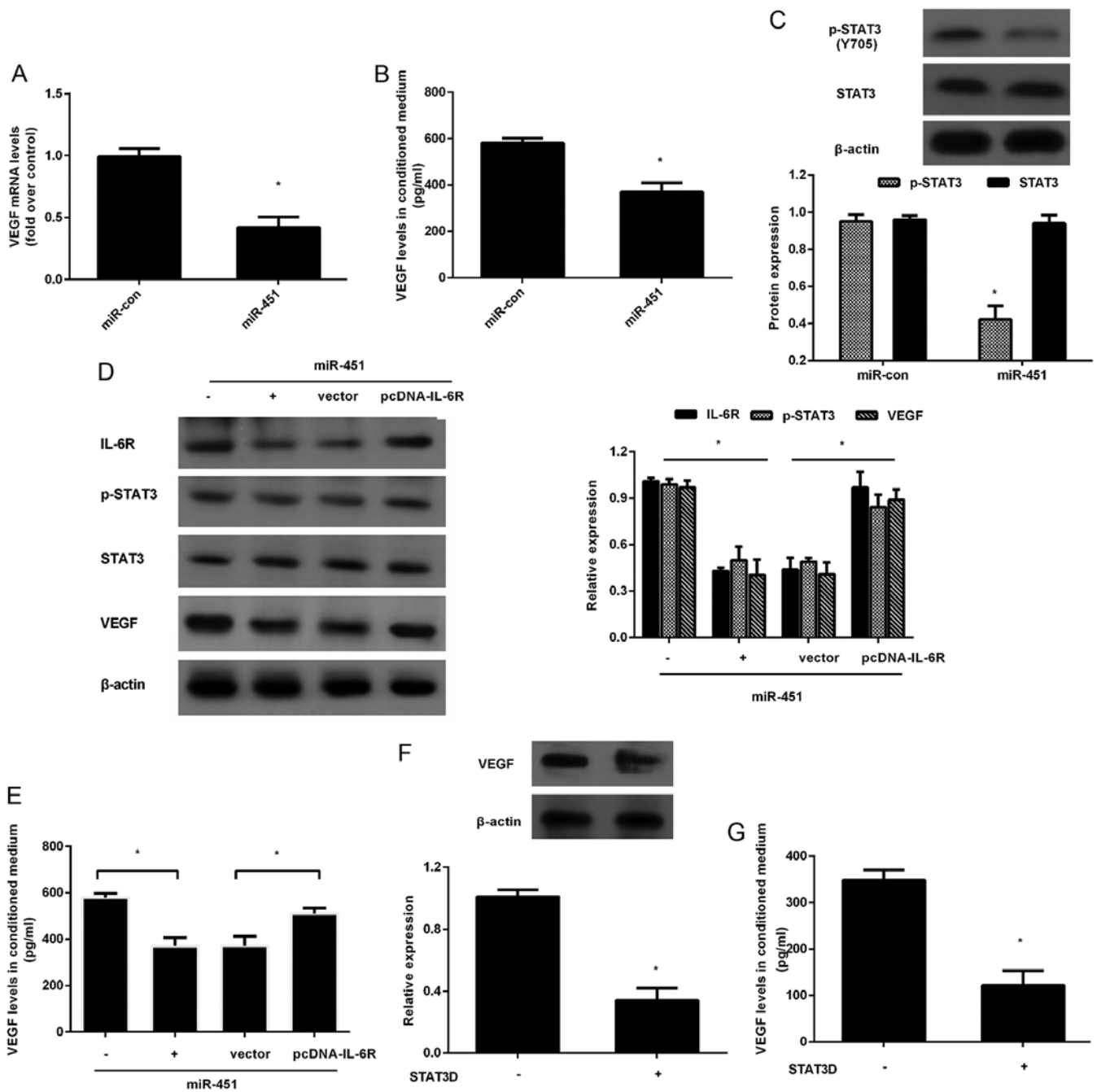
Figure 4. miRNA-451 (miR-451) suppressed vascular endothelial growth factor (VEGF) levels by blocking IL-6R-STAT3 signaling. (A) After transfection with miR-451, the microRNA (miRNA) levels of VEGF were detected. The corresponding effects on concentration of VEGF in (B) conditioned medium and (C) p-STAT3 levels were measured by ELISA and western blotting. The effects of IL-6 receptor (IL-6R) upregulation on the activation of (D) STAT3-VEGF pathway and (E) VEGF concentration were analyzed. Following transfection with dominant-negative STAT3 (STAT3D), (F) the protein levels and (G) the concentration of VEGF in HepG2 cells were assessed. *P<0.05.

Western blotting confirmed the obvious downregulation of p-VEGFR2 and p-ERK in HUVECs incubated with TCM of HepG2 cells that stably overexpressed miR-451 (Fig. 5A and B). Surprisingly, overexpression of IL-6R could remarkably restore the reduction of VEGF levels in TCM from miR-451-transfected HCC cells, which then ameliorated the inhibitory effect on the phosphorylation of VEGFR2 and p-ERK in HUVECs (Fig. 5C). Together, these results demonstrated that miR-451 could block the VEGFR2 pathway in HUVECs, which will lead to reduction in angiogenesis.

Discussion

Angiogenesis has an indispensable role in facilitating the development and progression of carcinoma (22,23). miR-451 is frequently decreased in various tumor types, including HCC (16). Substantial research has identified miR-451 as a tumor suppressor by exerting its restrained effect on cell proliferation, invasion and migration (16,17). Our previous study validated the stinking downregulation of miR-451 in HCC cells; its elevation notably suppressed HCC cell growth and invasion, indicating a potential role as tumor suppressor
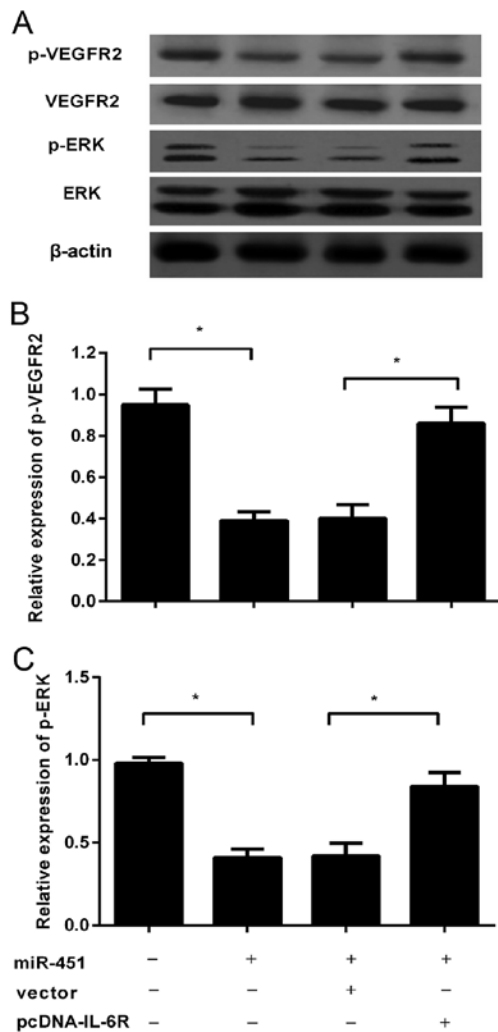
Figure 5. Incubation with tumor-conditioned medium (TCM) from miRNA-451 (miR-451)-transfected HepG2 cells dampened vascular endothelial growth factor (VEGF)2 signaling in human umbilical vein endothelial cells (HUVECs) by IL-6 receptor (IL-6R). (A) Following co-transfection with IL-6R and miR-451 in hepatocellular carcinoma (HCC) cells, the protein levels of VEGF receptor 2 (VEGFR2), p-VEGFR2, ERK and p-ERK in HUVECs were detected by western blotting. The corresponding quantitative analysis of (B) p-VEGFR2 and (C) p-ERK was performed by Quantity One. $^*P<0.05$.

in HCC (16). To date, nevertheless, its effect on angiogenesis in HCC remains undefined. In this study, we substantiated a finding that ectopic expression of miR-451 in HCC cells prominently inhibited cell proliferation, migration and capillary tube formation of HUVECs *in vitro*. Interestingly, its overexpression noticeably antagonized tumor growth and angiogenesis *in vivo*. Mechanism analysis reinforced that miR-451 suppressed VEGF production in HCC cells by targeting IL-6R-STAT3 signaling, as well as inhibiting the VEGFR2 signaling in HUVECs. Therefore, this research confirmed that miR-451 might act as a novel tumor suppressor in HCC by antagonizing angiogenesis through directly targeting IL-6R-STAT3-VEGF pathway.

Here, the IL-6R was identified as a candidate target of miR-451 using bioinformatics tools. IL-6R is known as a unique receptor of IL-6. Multiple research has documented the high expression of IL-6R and IL-6 in some tumors (12,24).

IL-6 has been proved to possess multiple biological function through IL-6R-mediated STAT3 signaling, such as cell growth and carcinogenesis (8). Increasing studies confirm that IL-6R exerts crucial roles in tumor angiogenesis (8,20). Ablation of IL-6R pronouncedly reduces oral squamous cell carcinoma (OSCC) growth and tumor angiogenesis by suppressing STAT3-mediated VEGF signaling, indicating a therapeutic approach against OSCC (20). It is intriguing to speculate that miR-451 may elicit its inhibitory effect on tumor angiogenesis by targeting IL-6R. Consistent with this hypothesis, miR-451 overexpression prominently mitigated IL-6R expression. Further luciferase activity assay reinforced that IL-6R was the direct target of miR-451. Elevation of IL-6R drastically attenuated the inhibitory effect of miR-451 on cell viability and migration of HUVECs. Importantly, IL-6R upregulation also antagonized the decrease in tube formation of HUVECs when incubated with TCM from miR-451-overexpressed HCC cells. Thus, based on these results we speculate that miR-451 might attenuate angiogenesis in HCC by targeting IL-6R.

Convincing evidence indicates that angiogenesis is pivotal for the growth and development of various cancer (19,23). During this process, tumor cells can secrete VEGF into the microenvironment to activate the vascular endothelial cells, which will subsequently facilitate tumor angiogenesis to meet tumor need for blood supply. VEGF has been reported to be an indispensable regulator for angiogenesis by regulating endothelial cell proliferation, migration and tube formation; blocking VEGF results in the regression of vascular network, ultimately suppressing tumor growth and metastasis (25,26). In the present study, miR-451 elevation significantly abrogated the expression of VEGF and secretion in HCC cells. Previous research has demonstrated that IL-6R can trigger angiogenesis by activating STAT3-VEGF pathway (8). STAT3 is constitutively activated in a variety of cancers and interrupting STAT3 signaling obviously attenuates tumor angiogenesis by VEGF production (27,28). Our previous results identified IL-6R as a direct target of miR-451. Further mechanistic analysis corroborated that the IL-6R-STAT3-VEGF signaling was notably restrained in HCC cells after miR-451 transfection. Upregulation of IL-6R counteracted the decrease of VEGF in miR-451-overexpressed HCC cells. Similarly to a previous study, blocking STAT3 signaling with STAT3D significantly decreased VEGF levels (28). Thus, the above data manifested that miR-451 might antagonize angiogenesis in HCC by targeting IL-6R-STST3-VEGF pathway.

A novel finding of this research is that miR-451 elevation in HCC cells inhibited VEGF levels in tumor microenvironment, which in turn suppressed the activation of VEGFR2 signaling in HUVECs. It is widely accepted that tumor-secreted VEGF can bind to and activate VEGFR2 signaling to promote vascular endothelial cell proliferation, migration and tube formation via ERK pathway (21,29). Blocking VEGFR2 can induce vessel normalization and survival benefit in mice bearing gliomas (30). Recently, suppressing VEGFR2 signaling has been proposed as a promising strategy for the clinical treatment of HCC (31). Accordingly, our research suggested miR-451 could abrogate the VEGF-VEGFR2 signaling, which finally abolished angiogenesis in HCC.

In conclusion, elevation of miR-451 in HCC cells saliently antagonized the viability, migration and tube formation of HUVECs by targeting IL-6R-STAT3-VEGF signaling. Importantly, its upregulation reduced tumor growth and angiogenesis of HCC *in vivo*. Moreover, overexpression of miR-451 in HCC cells also impaired VEGFR2 signaling in HUVECs. Therefore, miR-451 may act as a suppressor for angiogenesis of HCC by targeting IL-6R-STST3-VEGF signaling, indicating a promising therapeutic agent against HCC.

Acknowledgements

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