

## Original Article

# MicroRNA-195 regulates biological behavior of hemangioma cell lines by targeting HIF-1a

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Received September 21, 2015; Accepted December 6, 2015; Epub February 15, 2016; Published February 29, 2016

**Abstract:** MicroRNAs (miRNAs) have recently been reported play a crucial role in some tumors. In order to investigate the association of miR-195 with hemangioma, we investigate the expression of miR-195 in hemangioma tissues and its role in biological behavior of XTPS-1 and HemEChemangioma cancer cell lines. Quantitative qPCR results showed miR-195 was significantly down-regulated in hemangioma tissues compared with normal tissues. Over-expression of miR-195 was established by transfecting miR-195 mimics into XTPS-1 and HemECs cells, and its effects on the biological behavior of hemangioma cancer were studied using transwell assay, MTT and flow cytometry. Ectopic expression of miR-195 promoted cell proliferation of hemangioma cancer cells, while loss-of-function assay showed the opposite effect. Results indicated up-regulation of miR-195 significantly inhibited cell proliferation, invasion and resulted in apoptosis of the hemangioma cancer cells. Moreover, we demonstrated that miR-195 down-regulated HIF-1a expression by directly targeting the HIF-1a 3'-untranslated region. Thus, our findings suggested that miR-195 acted as a new tumor suppressor by targeting the HIF-1a gene and inhibiting cell proliferation and invasion of hemangioma.

**Keywords:** Hemangioma, HIF-1a, tumor suppressor

## Introduction

Hemangioma is the most common form of vascular tumor. For most patients, the lesions are small and pose no threat or potential for complication, but in some cases, hemangiomas grow dramatically and destroy tissue, impair function, or even threaten life. Hemangioma mostly occurred at the trunk skin surface, little known is about the mechanism of aberrant localized angiogenesis [1]. Proliferating hemangiomas are composed of densely packed cells with little connective tissue and barely discernible vessel lumen [2]. Recent studies suggested these regions contain a primitive cell type capable of giving rise to endothelial cells and pericytes. Clearly, understanding the cellular and biochemical mechanisms that control the growth and regression of blood vessels in hemangioma would provide critical insights into this particular tumor and angiogenesis in general [3]. Tissue of infantile hemangioma is com-

posed of diverse cell types unable to distinguish. Only certain kinds of cell type including hemangioma vascular endothelial cell (HemECs), hemangioma stem cells (HemSCs) and hemangioma mesenchymal stem cells (HemMSs) were used to current studies [4].

Much has been learned by studying the expression of angiogenic regulators during the proliferating, involuting, and involuted phases of hemangioma. Studies in recent years demonstrated that aberrant expression of a variety of miRNAs have been reported to be significantly involved in the regulation of vascular cell proliferation, which are essential process for the formation of hemangioma. MicroRNA (miRNA) are small non-coding RNAs that function as majors of post-transcriptional gene regulation in diverse biological processes, including cell proliferation, differentiation, migration, metabolism and apoptosis [5]. Dysfunction in miRNA regulatory networks resulting in aberrant gene expression

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in tumor initiation, development and metastasis [6, 7]. Additionally, hypoxia-inducible factor-1  $\alpha$  (HIF1 $\alpha$ ) and multiple miRNAs were reported closely associated with the formation of vessels in tumor [8]. HIF1 $\alpha$  protein concentration is correlated to cellular oxygen concentration. Hypoxic environment is essential for tumor cell growth, which leads to the accumulation of HIF1 $\alpha$ . The effects of HIF1 $\alpha$  are stimulation of erythropoiesis, glycolysis, angiogenesis, and vasodilation [9].

The epigenetic regulation of miRNAs is one of several mechanisms underlying carcinogenesis. MiRNA-195 is a critical regulator in cardiovascular disease and tumor growth, which has tumor suppressor properties in gastric cancer via down-regulating CDK6 and VEGF signaling and functions as a tumor suppressor by inhibiting CBX4 in hepatocellular carcinoma [10, 11]. Similar studies demonstrated that miR-195 expression can inhibit tumor cell proliferation and invasion by targeting of CDC42 in esophageal squamous cell carcinoma [12]. Theoretically, one kind of miRNA can regulate hundreds of target genes and a critical gene can be controlled by various miRNAs. Previous experiments have demonstrated multifaceted roles of miRNAs in cancer behaviors. The mechanisms of microRNA-195 in the formation of tumor growth have been studied for several years, but the potential role of microRNA-195 in hemangioma pathogenesis has not been investigated. Accordingly, this study examined the mechanisms underlying the relationship between the microRNA-195 and the proliferation of HemECs.

### Materials and methods

#### *Preparation of hemangioma specimens*

This study was approved by the Ethics Committee of the Kaifeng Central Hospital. Proliferating infantile hemangioma was surgically removed from a 34-years-old male patient who was referred to our department for a rapidly growing mass. Written informed consent was obtained from parents for all tissue obtained for the study. The clinical diagnosis of vascular neoplasm was confirmed by the Department of Pathology at the Kaifeng Central Hospital.

A total of 20 tissue specimens were obtained from our Hospital. All patients gave informed

consent prior to collection of specimens according to institutional guidelines. Tumor specimens, adjacent tissues (2.5 cm away from the tumor edge) and normal tissues were all collected from hemangioma at the time of radical cystectomy, and snap-frozen in the operating room immediately after surgery. The normal tissues (non-tumor tissues) were confirmed to be those surrounding tumor tissues and free of cancer cells by pathologic examination. The histopathology of the disease was determined by two pathologists according to the criteria of the World Health Organization. Clinical staging was done according to the Union for International Cancer Control classification.

#### *Cell culture and transfection*

Hemangioma vascular endothelial cell XTPS-1 was purchased from China Academia Sinica Cell Repository (Shanghai, China), in which XTPS-1 cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% fetal calf serum (Gibco, USA). Human dermal microvascular endothelial cells HEMC-1 were frozen in our hospital and cultured in DMED (Gibco, USA) supplemented with 10% fetal calf serum. All cells were cultured in a humidified incubator at 37°C in 5% CO<sub>2</sub>.

HemEC isolation from hemangioma tissue was performed as described previously [4]. Briefly, the hemangioma samples were rinsed in PBS, minced, and digested with 0.2% collagenase A at 37°C for 1 h. The tissue was homogenized and filtered through 100  $\mu$ m cell strainers to dissociate aggregates, and red blood cells were lysed by incubating the samples in NH<sub>4</sub>Cl. Next, the samples were filtered through a 40  $\mu$ m cell strainer to obtain a single-cell suspension. CD31<sup>+</sup> HemECs were isolated by FACS using anti-CD31 FITC antibodies and were planted on gelatin-coated 60 mm plates in EBM-2 medium supplemented with 20% heat-inactivated FBS, penicillin (100 units/ml) and streptomycin (100  $\mu$ g/ml). The cells were grown in humidified air containing 5% CO<sub>2</sub> at 37°C. Cells at passage 3 to 6 were used for experiments. The purity of the HemECs was >95% as determined by positive von Willebrand factor and CD31 expression, and by negative expression of vimentin (fibroblasts) and  $\alpha$ -actin (vascular smooth muscle cells) as previously described.

MiR-195 mimics and negative control RNA duplex (NC) were synthesized by Ribo Bio, Guangzhou, China. SiRNA duplexes with non-

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specific sequences were used as siRNA negative control (NC). Transfections were performed using Lipofectamine™ 2000 (Invitrogen, USA) according to the manufacturer's instructions. The final concentration of small RNAs was 50 nM.

### QPCR detection

Total RNA was extracted using TRIzol (Invitrogen, USA) according to the manufacturer's instructions. Reverse transcription and quantitative PCR were performed using the One Step PrimeScript miRNA cDNA Synthesis Kit (Takara, Dalian, China) by using the ABI 7500 Real Time PCR system (Applied Biosystems, Foster City, CA, USA). The primers were synthesized by Ribo Bio (Guangzhou, China). Moreover, the cycle threshold (Ct) value was used for our analysis ( $\Delta$ Ct), and we determined the expression of  $\beta$ -actin mRNA as internal controls to calculate the relative expression levels of miR-195, HIF-1a, VEGF via the  $2^{-\Delta\Delta Ct}$  method. The relative expression of miR-195 was normalized to the expression of  $\beta$ -actin.  $\beta$ -actin was amplified using the following primer: sense, 5'-CGTGAAAAGACCCAGATCA-3'; antisense, 5'-CACAGCCTGGATGGCTACGT-3'. Primer pairs for HIF-1a sense: CCGAATTGATGGGATATGAG; antisense: TCATGATGAGTTTTGGTCAGATG; Primer pairs for VEGF sense: GAGCCTTGCCTTGCTGCTCTAC; antisense: CACCAGGGTCTCGATTGGATG; the mRNA expression of VEGF and HIF-1a was normalized versus  $\beta$ -actin mRNA. The relative expression of miR-195 and HIF-1a was quantified with the  $2^{-\Delta\Delta Ct}$  method. Experiments were independently repeated three times.

### Plasmids, small interfering RNA and transfection

The miR-195 stem-loop (miR-195), inhibitors of miR-195 (miR-195-in) and negative control were purchased from RiboBio (Guangzhou, China). HIF-1a ORFs with 3'-UTR was amplified using PCR and subcloned into psiCHECK2 plasmids. Transfection in this assay performed as Lipofectamine 2000 reagent manufacturer's instructions. For HIF-1a depletion, small interfering RNA (siRNA- HIF-1a) was synthesized and purified by RiboBio (Guangzhou, China). Transfection of siRNAs were performed using lipofectamine 2000 (Invitrogen), according to the manufacturer's protocol.

### MTT

Cells were seeded at a density of 2000 cells per well in 96-well plates and cultured for 1, 2, 3, 4, 5 days after transfection. 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT; Sigma, St. Louis, MO) reagent (5 mg/mL) was added, followed by incubation for 4 h. Supernatant fractions were discarded and 150  $\mu$ L/well of DMSO added to terminate the reaction. Absorbance readings at 490 nm were obtained in triplicate using a spectrophotometric plate reader (Thermo Scientific, Waltham, MA).

### Apoptosis assay

Cells (XTPS-1) apoptosis was detected by Guava Nexin Reagent manufacturer's protocol (Millipore, USA) after transfection for 48 h, which containing Annexin V-PE and 7-AAD dye. Transfected XTPS-1 cells were washed and resuspended in  $1\times$  binding buffer at a concentration of  $1\times 10^5$  cells per ml. Then cells ( $1\times 10^5$ ) were incubated with 20  $\mu$ L of Annexin V-PE and 7-AAD for 30 min at room temperature in the dark. Briefly, about 5,000 cells were analyzed using flow cytometry (FCM), which recorded the number of cells through the indicated gates. Experiments were independently repeated three times.

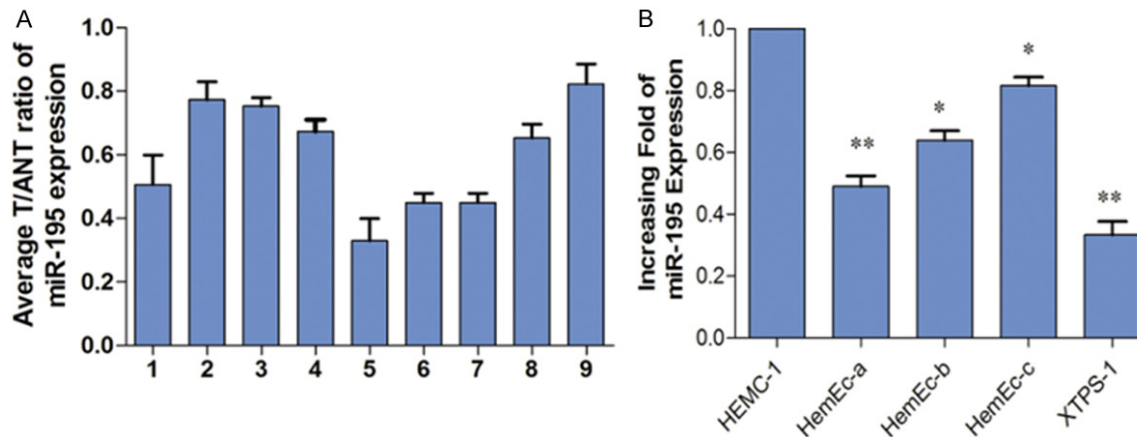
### Cell cycle analysis

Cell cycle distribution was analyzed by flow cytometry (Beckman Coulter, Brea, CA). After treatment, the cells were trypsinized, centrifuged at  $1,000\times$  g for 5 min, collected and washed with ice-cold PBS. Next, the cell pellets were resuspended and fixed with cold 70% ethanol overnight. After another wash with PBS, the cell pellets were resuspended in 1 mL of staining solution containing propidium iodide (PI, 50  $\mu$ g/mL), DNase-free RNase (100  $\mu$ g/mL) and Triton-100 (0.3%, Bioengineering Corporation, Shanghai, China). Finally, the cells were incubated at 37°C for 30 min in the dark before analysis. The fraction of the cell population in each phase of the cell cycle was determined as a function of the DNA content using flow cytometry analysis.

### Western blot

Western blot analysis using antibodies against VEGF, HIF-1a and GAPDH was performed on

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**Figure 1.** Expression of miR-195 in hemangioma clinical specimens and cell lines. A. Relative miR-195 expression levels in 9 paired primary hemangioma tissues and the matched tumor adjacent normal tissues (ANT) from the same patient were detected by PCR analysis. B. Real-time PCR analysis of miR-195 expression in hemangioma cell line XTPS-1 and hemangioma vascular endothelial cells (HemEC) isolated from 3 of 9 primary hemangioma tissues, which were termed as HemEC-a, HemEC-b and HemEC-c respectively. Human dermal microvascular endothelial cells HEMC-1 were used as control. Experiments were repeated at least three times. Each bar represents the mean of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

extracted proteins as previously described. Protein concentrations were detected with the Bradford assay (Bio-Rad) and equal amounts of protein were analyzed by SDS-PAGE. Gels were electro-blotted onto polyvinylidene difluoride-membranes (Millipore, Billerica, WI, USA). The proteins were visualized by ECL, and the intensity of the signal was quantified by scanning laser densitometry. GAPDH was served as a loading control. Experiments were independently repeated three times.

### Statistical analysis

All data were expressed as the mean  $\pm$  SD with  $n = 3$  for each sample for all of the paired statistical comparisons. The analysis of variance (ANOVA) test followed by Tukey's t-test was performed, and a  $P$  value less than 0.05 was considered statistically significant.

### Results

#### *MiR-195 expression was downregulated in hemangioma tissues and hemangioma cell lines*

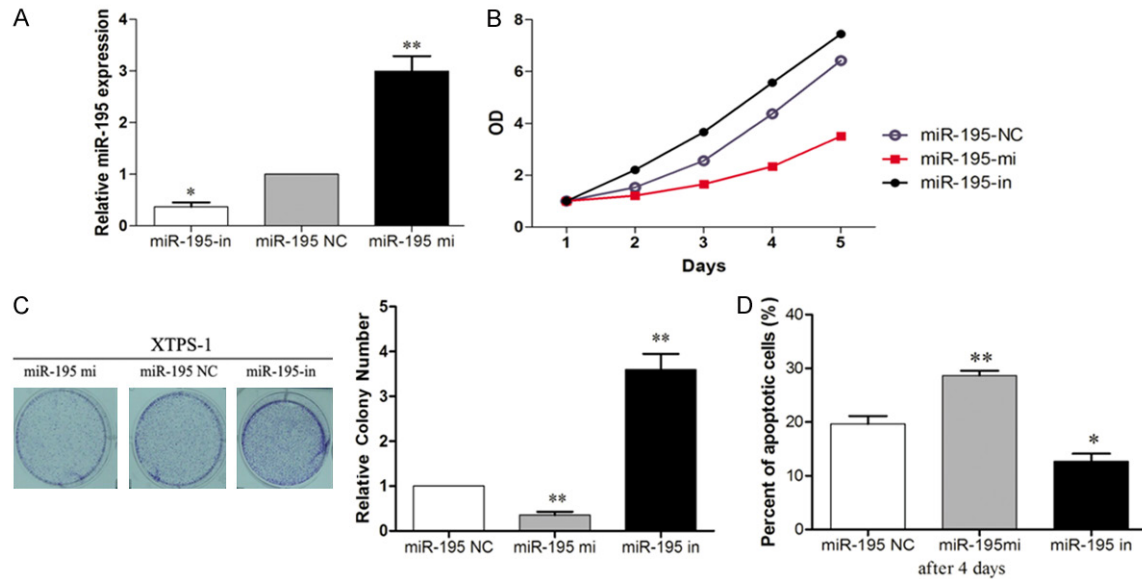
The expression pattern of miR-195 in human hemangioma has not been analyzed. To testify the correlation between miR-195 and hemangioma, the expression level of miR-195 was measured by qPCR in hemangioma tissues and normal tissues. As shown in **Figure 1A**,

compared with their non-cancerous counterparts, it was observed that miR-195 expression levels were lower in hemangioma tissue, it was also shown that miR-195 was down-regulated in hemangioma cell lines compared with HEMC-1 (**Figure 1B**). Together, we conclude that miR-195 was abnormally down-regulated in hemangioma tissues and hemangioma cell lines.

#### *Over expression of miR-195 suppressed cell proliferation and promoted apoptosis of hemangioma cells*

The effect of miR-195 in terms of proliferation of hemangioma cells were analyzed at the 48<sup>th</sup> after transfection. We transfected XTPS-1 cells with miR-195 mimics, miR-195 inhibitors and negative control group respectively. The transfection efficiency of miR-195-mi and miR-195-in was validated by qPCR (**Figure 2A**). MTT and colony formation assays were used to further assess the potential effect of miR-195 on tumorigenesis, our data indicated that proliferation of XTPS-1 cells was decreased when cells were transfected with miR-195 mimics, while the miR-195-in showed the opposite effect (**Figure 2B** and **2C**). Using flow cytometry assays, we found that miR-195 overexpression increased the percentage of apoptotic cells, the opposite result was obtained when the cells were treated with miR-195-in (**Figure 2D**). Together our results suggest that miR-195

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**Figure 2.** MiR-195 upregulation inhibited cell proliferation and induced apoptosis of hemangioma cell. A. Validation of miR-195 expression levels after transfection by PCR analysis. B. MTT assays revealed that upregulation of miR-195 inhibited growth of XTPS-1 cell line. C. Representative micrographs (left) and quantification (right) of crystal violet-stained cell colonies. D. Flow cytometric analysis of the indicated XTPS-1 cells transfected with NC or miR-195-in. Each bar represents the mean of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

reduced hemangioma cell tumorigenicity in vitro.

*MiR-195 directly restrained the expression of HIF1a through direct targeting of HIF1a mRNA 3'UTR and altered levels of proteins related to cell proliferation and cycle in hemangioma cells*

Potential target of miR-195 was predicted using bioinformatics methods. HIF-1a was selected as the target for further analysis. XTPS-1 cells were transiently transfected with miR-195 mimics, miR-195-in and NC. We observed that miR-195 was capable of down-regulating HIF-1a protein expression (**Figure 3E**). To gain insight into the mechanism by which miR-195 inhibits HIF-1a, we identified the miR-195 binding site in the HIF-1a mRNA 3'UTR (**Figure 3A**) and constructed psiCHECK-2-HIF-1a-wild type and psiCHECK-2-HIF-1a-mut type plasmids. Results of luciferase reporter assays showed that the co-transfection of miR-195 markedly increased the firefly luciferase activity of psiCHECK-2-HIF-1a-wild but failed to influence the luciferase activity of psiCHECK-2-HIF-1a-mut in XTPS-1 cells (**Figure 3B**). Meanwhile, XTPS-1 cells transfected with miR-195-in resulted in suppressing firefly luciferase activity of the wild-type reporter but unaffected the mutant report-

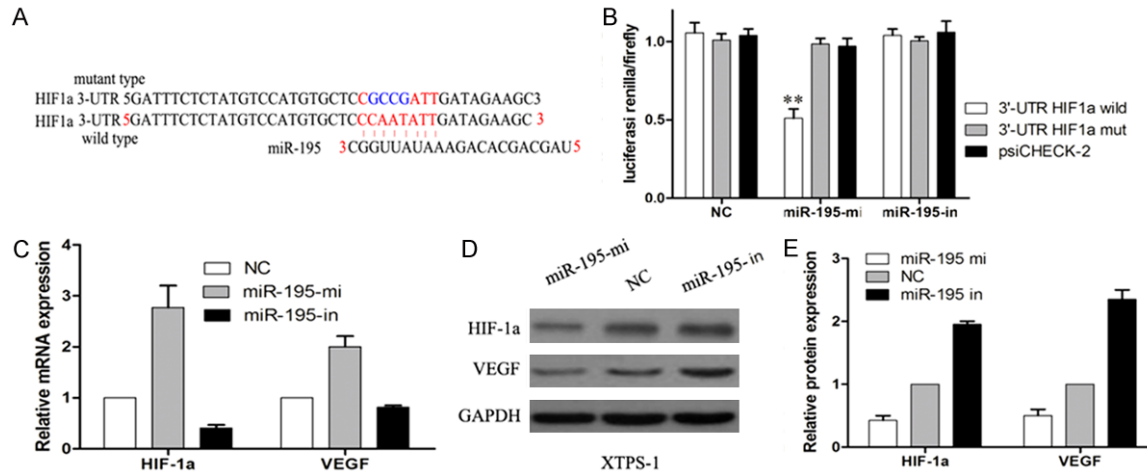
er (**Figure 3B**). In summary, our data indicate that miR-195 directly attenuated the expression of HIF-1a by targeting of its mRNA 3'UTR in hemangioma cell lines.

Previous studies revealed that enhancing transcriptional activity of HIF-1a resulting in upregulation of VEGF, which was closely related to cell proliferation [13]. To investigate the mechanism underlying cell proliferation, we tested the expression of downstream regulators VEGF of HIF-1a. Results of real-time PCR and Western blotting analysis showed that VEGF and HIF-1a (mRNA and protein) levels were downregulated in miR-195-transfected XTPS-1 cells, compared to NC-transfected cells, while miR-195-in showed the opposite results (**Figure 3C** and **3D**). Altogether, our results indicated that miR-195 regulated expression of HIF-1a, and then functionally modulated cell proliferation, HIF-1a and VEGF, thus relevant to cell proliferation and apoptosis.

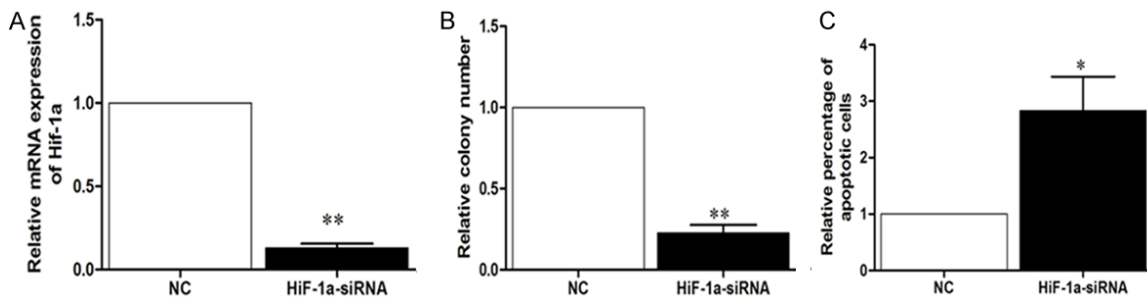
*HIF-1a is involved in miR-195-induced proliferation of hemangioma cells*

To further understand the role of HIF-1a on proliferation mediated by miR-195, we transfected XTPS-1 cells with HIF-1a-siRNA. As showed in **Figure 4A**, results of Western blot analysis

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**Figure 3.** MiR-195 decreased HIF-1a expression by directly targeting the HIF-1a 3'-UTR and altered levels of proteins related to cell proliferation in XTPS-1 cells. **A.** Predicted miR-195 target sequence in the 3'-UTR of HIF-1a (HIF-1a-3'-UTR) and positions of four mutated nucleotides (blue) in the 3'-UTR of HIF-1a (HIF-1a-3'-UTR-mut). **B.** Luciferase reporter assay of the indicated cells transfected with the psiCHECK-2-HIF-1a-3'-UTR reporter or psiCHECK-2-HIF-1a-3'-UTR-mut reporter and miR-195 or miR-195-in with 50 nM oligonucleotides. **C.** Real-time PCR analysis of expression of VEGF and HIF-1a in indicated XTPS-1 cells. **D.** Western blotting analysis of HIF-1a expression in cells transfected with miR-195-mi or the miR-498-in. GAPDH served as the loading control. **E.** Analysis of relative expression of protein in **Figure 2D**. \* $P < 0.05$ , \*\* $P < 0.01$ .



**Figure 4.** HIF-1a upregulation is required for miR-195-induced pro-apoptotic role and suppression of proliferation in hemangioma cells. **A.** QPCR analysis verified that silencing HIF-1a effectively decreased the expression of HIF-1a in miR-195-transfected XTPS-1 cells. **B.** Analysis of relative colony number in XTPS-1 cells after transfection with HIF-1a-siRNAs, transfection with HIF-1a suppressed cell colonies formation. **C.** Flow cytometry detected the percentage of apoptotic cells after transfecting with Hif-1a-siRNA. Each bar represents the mean of three independent experiments. \* $P < 0.05$ , \*\* $P < 0.01$ .

revealed that the HIF-1a expression in miR-195-transfected XTPS-1 cells was decreased after transfected with HIF-1a-siRNA. Colony formation assays showed that the treatment with HIF-1a-siRNA was able to reverse the miR-195-decreased proliferation (**Figure 4B**), suggesting that miR-195 suppresses the proliferation of ovarian cancer cells by upregulating HIF-1a. Additionally, HIF-1a-siRNA transfected XTPS-1 cells induced the cell apoptosis compared with NC group. Therefore, our results demonstrate that miR-498 was able to inhibit the proliferation and promote the apoptosis of

hemangioma cells through direct targeting HIF-1a.

### Discussion

The key finding of the current study is that miR-195 expression was markedly downregulated in hemangioma tissues and hemangioma vascular endothelial cell, XTPS-1 cells as compared with that in the matched tumor adjacent normal tissues and human dermal microvascular endothelial cell HEMC-1 respectively. Our result revealed that ectopic expression of miR-195 could decrease the cell proliferation of heman-

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gioma, while miR-195-in enhanced this effect. Moreover, overexpression of miR-195 could induce the apoptosis of hemangioma cell line. Result of dual-luciferase assay showed that HIF-1a was a direct target of miR-195. Further experiment showed that upregulation of miR-195 in hemangioma cells led to downregulation of HIF-1a and VEGF [14]. Taken together, these findings demonstrated that miR-195 may play an important role in carcinogenesis and progression of hemangioma.

Numerous studies indicated that MicroRNAs (miRNAs), a class of small non-coding RNAs, potentially play critical roles in cell proliferation, cycle, differentiation, angiogenesis, invasion and migration of various types of human cancers [15]. MiRNAs regulate gene expression and are proposed as potential novel targets for anti-cancer therapies [16, 17]. However, it was uncertain whether dysregulation of miR-195 was associated with the progression of hemangioma. In this study, our results revealed that miR-195 was significantly down-regulated in hemangioma, resulting in promotion of cell proliferation and decreasing of apoptotic cells. Above all results indicated that miR-195 function as a candidate tumor suppressor in the pathogenesis of hemangioma in vitro.

The amount of HIF-1a protein is correlated to cellular oxygen concentration. In hypoxic conditions, HIF1a is not degraded and accumulates in the cellular nucleus [18]. HIF-1a protein is a member of the forkhead transcriptional factor family, which is a stimulation of erythropoiesis, glycolysis, angiogenesis, and vasodilation [14]. Additionally, HIF-1a protein is a critical regulator of a series gene expression, including VEGF, PDGF and PFKL etc. [19]. Previous studies indicated that VEGF protein level always associated with Hif-1a protein concentration [20, 21]. According to bioinformatics prediction, the tumor suppressor Hif-1a was indicated as a theoretical targeted gene of miR-195. Result of Western blotting analysis showed that overexpression of miR-195 resulted in the decrease expression of Hif-1a protein. VEGF expression level at mRNA and protein level was detected to verify downregulation of Hif-1a. Additionally, VEGF was a critical regulator of cell cycle and proliferation. Together result of MTT assays, colony formation assays and flow cytometry analysis, our data indicated that miR-195 exerts its functions by down-regulating expression of Hif-1a, which afterward causes down-

regulating of expression of VEGF, affecting biological behavior of hemangioma cell lines, including cell proliferation, apoptosis. Furthermore, Hif-1a-silenced in XTPS-1 cells had similar effect on cell proliferation and apoptosis, suggesting that direct down-regulation of Hif-1a is required for miR-195-induced transformation of hemangioma cell line.

In summary, the current study provides that an important link between miR-195-mediated proliferation of hemangioma cells and down-regulation of Hif-1a. Our findings revealed a novel role of miR-195 in the regulation of hemangioma cells biological behaviors. All the results indicated that miR-195 was a potential anti-oncomiR for hemangioma therapy.

### Acknowledgements

This work was supported by Department of Thoracic and Cardiovascular Surgery, Intensive Care Unit. All authors designed the study together, performed the experiment together, analyzed the data and wrote the paper; all authors approved the final manuscript.

### Disclosure of conflict of interest

None.

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