Cellular and Molecular Biology
E-ISSN : 1165-158X / P-ISSN : 0145-5680
www.cellmolbiol.org

*Original Research*

# Down-regulation of miR-556-3p inhibits hemangioma cell proliferation and promotes apoptosis by targeting VEGFC

Wen Jin[1#], Liang Chen[2#], Fengyun Gao[1], Mei Yang[1], Yan Liu[1], Bo Wang[1*]

[1] Department of Medical Nursing, Tongling polytechnic College, China
[2] Department of hyperbaric oxygen, The Second People's Hospital of Hefei, China

[*]**Correspondence to:** jia1982yun@163.com

**Received** May 25, 2020; **Accepted** June 30, 2020; **Published** July 31, 2020

[#] These authors contributed equally to this work as co-first author.

**Doi:** http://dx.doi.org/10.14715/cmb/2020.66.5.34



**Abstract:** The purpose of this study was to determine the effect of microRNA (miR)-556-3p on cell proliferation and apoptosis of hemangioma-derived endothelial cells (HemECs). qRT-PCR was used to compare the expressions of miR-556-3p in HemECs and normal cells. The target gene was identified using dual-luciferase reporter assay. Cell proliferation was measured with MTT assay, while western blotting was used to assay VEGFC expression levels. Apoptosis was assayed with FITC Annexin V Apoptosis assay kit. miR-556-3p was overexpressed in HemECs. Transfection with miR-556-3p inhibitor resulted in decreased proliferation of HemECs ($p<0.05$). The expression of the target gene of miR-556-3pi.e. VEGFC was upregulated when miR-556-3p inhibitor was transfected. The transfection also resulted in increased apoptosis. In HemECs, miR-556-3p is overexpressed and VEGFC expression is low. Thus, miR-556-3p or VEGFC might be potential targets for treatment of angiosarcoma.

***Key words:*** Angiosarcoma; HemECs; miR-556-3p; VEGFC; Proliferation; Apoptosis.

## Introduction

Infantile hemangioma (IH), the most diagnosis tumor of infancy, typically appears in the second week of life (1). This tumor is usually benign, and may be ignored because most patients usually recover, but some patients with serious complications require treatment (2). The typical life span of benign hemangiomas comprises three phases: proliferating phase, which leads to involuting phase, and finally to involuted phase (3, 4). Pharmacological inhibition of VEGF signaling could result in growth suppression and apoptosis in HemECs. However, the mechanisms underlying hemangioma growth is still not completely understood.

MicroRNAs (miRs) which were first discovered in 1993 in the nematode *Caenorhabditis elegans,* are a class of small non-coding RNAs (approx. 22 nucleotides in length) which play important roles in a broad range of physiological and pathological processes (5). The miRNA regulate target gene expression through partial complementary binding to 3′-untranslated region (UTR), and their expressions have been found to be specific in different cancer cells(6). Increasing evidence have shown that dysregulation of miRNAs usually indicates tissue abnormality. Previous reports have demonstrated that miR-21a-3p inhibitor blocked endothelial cell tumor growth by targeting Nox-4 in a mouse model (7). In contrast, miR-424 inhibitor promoted cell proliferation in hemangioma tumor cells(8). It has been reported that miR-143 regulated the biological behaviors of hemangioma cells in different origins, inducing migration and angiogenesis and increasing the permeability of cells (9). Moreover, miR-143 inhibited tumor cell proliferation in multiple cancer types such as esophageal squamous cell carcinoma and glioblastoma (10). In spite of these studies, there is need for more investigation on the effect of miR-556-3p in the growth of hemangioma.

Vascular endothelial growth factor C (VEGF-C), belongs to the VEGF gene family which is thought to induce lymph angiogenesis, leading to lymphatic vessel invasion and lymph node metastasis (11). It promotes angiogenesis and endothelial cell proliferation, and affects the permeability of blood vessels. (12). In a previous study, Xia *et al*. reported that upregulation of VEGF-C expression was associated, not only with poor prognosis in esophageal cancer patients, but also lymphatic involvement and poor prognosis in patients with esophageal squamous cell carcinoma (11). However, not much is known about the relationship between IH and VEGFC.

The present study was aimed at investigating the effect of miR-556-3p on HemECs cells, and its effect on the cell proliferation and apoptosis. Furthermore, the identity of the direct target gene involved in the effect of miR-556-3p on HemECs was investigated.

## Materials and Methods

### Chemicals and drugs

Dimethylsulfoxide(DMSO), PI and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

were purchased from Sangon (Shanghai, China). Penicillin, streptomycin, fetal bovine serum (FBS) and EBM-2 medium were bought from Sigma Aldrich (Carlsbad, CA), while FITC-Annexin V/PI apoptosis detection kit and cell cycle analysis kit were obtained from BD Biosciences. Antibodies against VEGFC were obtained from Cell Signaling Technology (CST, Danvers, MA, USA). Antibody for GAPDH was product of Santa Cruz Biotechnology (Santa Cruz, CA, USA). Dual-Luciferase Reporter Assay kit was purchased from Promega (Wisconsin, WI, USA).

**HemECs cells culture**

The HemECs and normal cells were supplied by American Tissue Culture Collection (ATTC, UK). The cells were cultured in Endothelial Basal Medium-2 with 10% fetal bovine serum., and then sub-cultured in 0.05% trypsin-EDTA solution (Invitrogen Life Technologies). In this study, HemEC cells were used at passages 3–8.

**qRT-PCR analysis**

The expression of miR-556-3p was assayed using qRT-PCR analysis. Total RNA was extracted from cells using TRIzol reagent in line with the manufacturer's instructions (Invitrogen Life Technologies, Carlsbad, CA, USA). The RNA was reverse-transcribed into cDNA using Taqman miRNA Reverse Transcription kit, with U6 as an endogenous control for comparison.

**Cell transfection**

The HemECs were transfected with miR-556-3p inhibitor (50 nM) using Lipofectamine 2000 (Invitrogen Life Technologies) in accordance with the manufacturer's instructions. Thereafter, the cells were cultured and prepared for use in subsequent experiments.

**Luciferase assays**

The pEZX Vectors used were purchased from Gene Copoeia Co. (Guangzhou, China). Cells were cultured in 24-wellplates at a density of 1 x $10^5$/well in triplicate, and co-transfected with pEZX-VEGFC-30-UTR with VEGFC ORFs and 100 nM of miR-556-3p using Lipofectamine 2000 Reagent. Luciferase activity was assayed using Dual-Luciferase Reporter Assay kit.

**MTT assay**

The effect of miR-556-3p on the proliferation of human HemECs cells was measured with MTT assay. The cells were seeded at a density of 1x$10^4$ cells per well in 96-well plates, and transfected with miR-556-3p inhibitor. Following incubation, the supernatant was removed, and MTT solution (100 µl) was added to the cells and incubated for an additional 4 h. Then, 150 ml DMSO was added, and the absorbance of each formazan solution was read at 570 nm in a micro-plate reader (Thermo Scientific).

**Flow cytometry**

The cells were first treated with Annexin V-FITC (25 µg/ml; Beyotime Biotechnology, Hainen, China). Then, PI (25 µg/ml) was added and incubated for 10 min at room temperature in the dark. Then, flow cytometry was used to measure the apoptosis by determining the relative amount of Annexin V–FITC-positive and PI-negative cells.

**Western blot analysis**

Following transfection, total protein was extracted from the HemECs cells using standard methods. Equal amount of proteins were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and then transferred onto a polyvinylidene difluoride (PVDF, Thermo Fisher Scientific, Inc.) membranes. The membranes were then incubated with antibodies against VEGFC (#2445, 1:1000, Cell Signaling Technology,) and GAPDH. After washing with PBST, the membrane was incubated with secondary antibodies, and visualized with ECL kit (Beyotime, Shanghai, China). The internal control GAPDH.

**Statistical analysis**

Results are presented as mean ± S.D (n =3). Differences amongst groups were analyzed using a one-way analysis of variance (ANOVA) followed by Dunnett's test. Differences were considered statistically significant at $p < 0.05$.

## Results

**miR-556-3p was overexpressed in HemECs**

Compared with non-tumor tissues, miR-556-5p was significantly upregulated at the mRNA levels in all 3 tumor cells samples ($p < 0.05$), as shown in Figure1.

Following transfection with miR-556-3p inhibitor, cell proliferation was measured using MTT assays. The proliferation of miR-556-3p inhibitor group was significantly lower than that of any other group ($p< 0.05$). These results suggest that downregulation of miR-556-3p inhibited the proliferation of HemECs cells.

**MiR-556-3p directly targets VEGFC by binding to its 3-UTR**

As predicted by targetSCAN (http://www.targetscan.org/vert_71/), VEGFC may be a target of miR-556-3p. To determine the target relationship between miR-556-3p and VEGFC, Luciferase assays was performed. As shown in Figure 3, VEGFC was the target gene in HemEC cells.

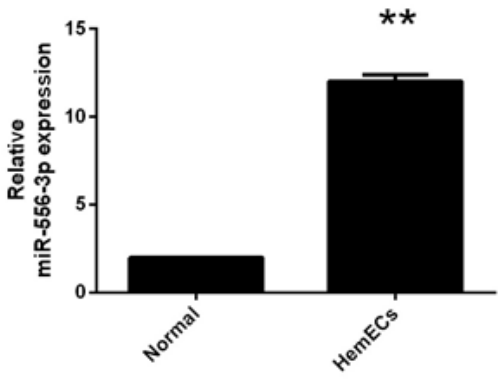


**Figure 1.** Expression of miR-556-3p was upregulated in human HemECs cancer cell lines (A). (B). Each bar represents the mean of three independent assays. **$p < 0.05$.Transfection with miR-556-3p inhibitor decreased the proliferation of HemFCs.

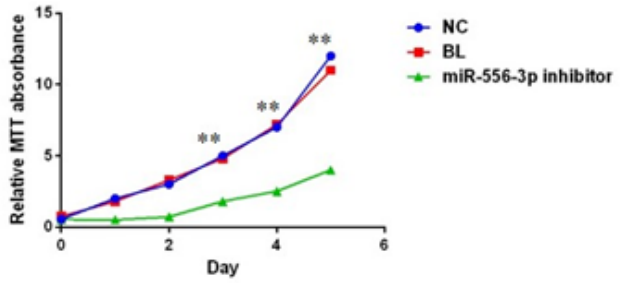


**Figure 2.** Down-regulation of miR-556-3p inhibited the proliferation of HemECs cancer cells.NC mean normal control, BL mean blank control. **$p$<0.05 VS miR-556-3p inhibitor.

## Transfection with miR-556-3p inhibitor increased apoptosis of HemECs

Flow cytometry was used to investigate further, the influence of miR-556-3p on HemECs cells. As shown in Figure 3, transfection with miR-556-3p inhibitor significantly increased apoptosis of HemECs ($p$< 0.05).

## VEGFC expression was up-regulated after transfection of miR-556-3p inhibitor

There was significant decrease in VEGFC expression in the miR-556-3p inhibitor group, relative to the blank control and normal control groups. However, VEGFC expressions in blank control and normal control groups were comparable. These results indicate that downregulation of miR-556-3p could increase VEGFC expression. Thus, VEGFC may be a target gene for miR-556-3p.

## Discussion

It is well known that protein-coding genes are involved in the development of human tumors. There is growing evidence indicating that disorders in non-coding genes, particularly microRNAs (miRNAs), also contribute to tumorigenesis(13). It has been predicted that about one-third of human protein-coding genes are regulated by miRNAs. In fact, miRNAs have been implicated in the control of various biological processes including tumor development, cell proliferation, apoptosis and differentiation. There is growing evidence that miRNAs may act as oncogenes or tumor suppressors(14-16). This study hoped to determine if the miRNA-556-3p is an oncogene in IH, and regulate the IH process via targeting VEGFC.

Currently, propranolol, a non-selective β-adrenoceptor blocker, is the preferred treatment for problematic proliferative his, and has been identified as a safe and effective treatment option that significantly reduces IH tumor size, leading to significant shortening of the natural processes of IH without any observable adverse effects(17). In addition, propranolol has produced marked and rapid effects, especially for IHs involving dyspnea, eyelid occlusion, hemodynamic lesions or ulcers. However, the mechanism of action of propranolol has not yet been fully elucidated. The mechanism of action of propranolol may involve a combination of events, rather than a single event (18, 19).

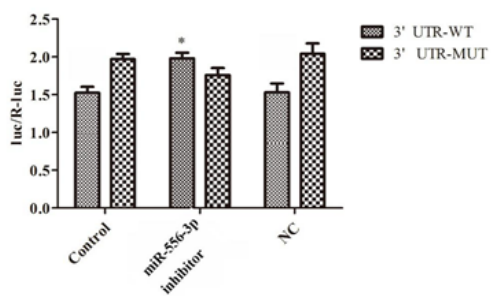


**Figure 3.** miR-556-3p targeted 3'-UTR of VEGFC.

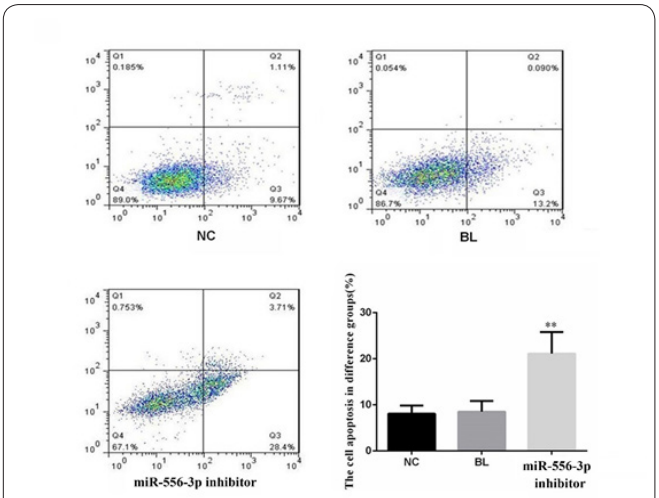


**Figure 4.** Effect of miR-556-3p inhibitor transfection on apoptosis. Transfection increased apoptosis in HemECs, when compared with NC and BL.NC mean normal control, BL mean blank control. **P <0.05.

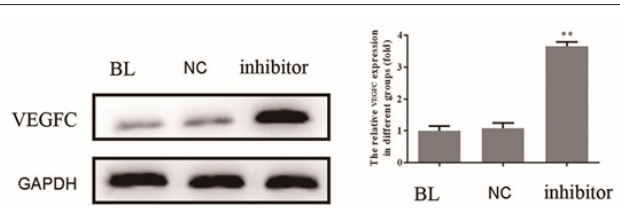


**Figure 5.** VEGFC expression was up-regulated after transfection of miR-556-3p inhibitor.Down-regulation of miR-556-3p significantly increased VEGFC expression (**$p$<0.05). NC mean normal control, BL mean blank control.

In order to determine the function of miR-556-3p in HemECs cells, the miR-556-3p expression was first determined using qRT-PCR, and the results showed overexpression of miR-556-3p in HemECs cells. In further studies, VEGFC was predicted as the target gene for miR-556-3p, using target SCAN, and confirmed with Luciferase assays. The VEGFC expression was also determined with Western blotting. At last, it was found that down-regulation of miR-556-39 promoted HemECs cell apoptosis, while MTT assay showed that cell proliferation was inhibited.

These results showed that miRNA-556-3p was up-regulated in all HemECs, which was in line with prediction. This means that the target genes may be used in the diagnosis and treatment of cancer according to its different roles and expression level. In this study, it was speculated that in IH cells, if the miRNA targeted a specific gene, its expression level was supposed to regulate the expression of the target gene and influence cell proliferation and apoptosis.

In conclusion, the findings from this study indicate that downregulation of miR-382 inhibited the expression of VEGFC. This provides a new insight and mechanism for explaining the function of VEGFC in IHs. In the future, research will be conducted to sufficiently justify this mechanism in IHs at the organism level.

**Acknowledgments**
The work was supported by Anhui provincial quality engineering project for colleges and universities (2019cxtd043,2018jyxm0910,2018ylzy183).

**Conflicts of interest**
There are no conflicts of interest in this study.

**Author's contribution**
All work was done by the author named in this article and the authors accept all liability resulting from claims which relate to this article and its contents. The study was conceived and designed by Bo Wang; Wen Jin, Liang Chen, Fengyun Gao, Mei Yang, Yan Liu, Bo Wang collected and analysed the data; Wen Jin and Liang Chen wrote the text and all authors have read and approved the text prior to publication. Wen Jin and Liang Chen contributed equally to this work and should be considered as co-first authors.

**References**

1. Boye E, Yu Y, Paranya G, Mulliken JB, Olsen BR, Bischoff J. Clonality and altered behavior of endothelial cells from hemangiomas. J Clin Invest 2001; 107: 745-752.
2. Mabeta P, PepperMS. Inhibition of hemangioma development in a syngeneic mouse model correlates with bcl-2 suppression and the inhibition of Akt kinase activity. Angiogenesis2012; 15: 131-139.
3. Gnarra M, Behr G, Kitajewski A, Wu JK, Anupindi SA, Shawber CJ, et al. History of the infantile hepatic hemangioma: From imaging to generating a differential diagnosis. World J Clin Pediatr2016; 5: 273-280.
4. Walter JW, North PE, Waner M, Mizeracki A, Blei F, Walker JW, et al. Somatic mutation of vascular endothelial growth factor receptors in juvenile hemangioma. Genes Chromosomes Cancer 2002; 33: 295-303.
5. Lee RC, Feinbaum RL, Ambros V.The C. elegans heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. Cell 1993; 75: 843-854.
6. Volinia S, Calin GA, Liu CG, Ambs S, Cimmino A, Petrocca F, et al. A microRNA expression signature of human solid tumors defines cancer gene targets. Proc Natl Acad Sci U S A 2006; 103: 2257-2261.
7. Gordillo GM, Biswas A, Khanna S, Pan X, Sinha M, Roy S, et al. Dicer knockdown inhibits endothelial cell tumor growth via microRNA 21a-3p targeting of Nox-4. J Biol Chem 2014; 289: 9027-9038.
8. Nakashima T, Jinnin M, Etoh T, Fukushima S, Masuguchi S, Maruo K, et al. Down-regulation of mir-424 contributes to the abnormal angiogenesis via MEK1 and cyclin E1 in senile hemangioma: its implications to therapy. PLoS One2010; 52: 14334.
9. Huang C, Huang J, Ma P, Yu G. microRNA-143 acts as a suppressor of hemangioma growth by targeting Bcl-2. Gene 2017; 628: 211-217.
10. Fu TG, Wang L, Li W, Li JZ, Li J. miR-143 inhibits oncogenic traits by degrading NUAK2 in glioblastoma. Int J Mol Med 2016; 37: 1627-1635.
11. Kimura Y, Watanabe M, Ohga T, Saeki H, Kakeji Y, Baba H, et al. Vascular endothelial growth factor C expression correlates with lymphatic involvement and poor prognosis in patients with esophageal squamous cell carcinoma. Oncol Rep 2003; 10: 1747-1751.
12. Xia H, Shen J, Chen S, Huang H, Xu Y, Ma H. Overexpression of VEGF-C correlates with a poor prognosis in esophageal cancer patients. Cancer Biomark 2016; 17: 165-170.
13. Calin GA, Croce CM. MicroRNA signatures in human cancers. Nat Rev Cancer 2006; 6: 857-866.
14. Schratt GM, Tuebing F, Nigh EA, Kane CG, Sabatini ME, Kiebler M, et al. A brain-specific microRNA regulates dendritic spine development. Nature 2006; 439: 283-289.
15. Wu LN, Gao YX, Ruan JL, Yi LR, Sun L, Piao CN, et al. Effects of a high-radon hot spring on the cell cycle and its regulatory proteins in the peripheral blood lymphocytes of surrounding residents. Acta Medica Mediterr 2019; 35: 1831-1836.
16. Shi YQ, Qiu JW, Qian JB, Wang YM, Ge ZM. The correlation between mirna-10a expression and serum tnf-a and il-6 levels in patients with inflammatory bowel disease and its diagnostic value. Acta Medica Mediterr 2019; 35: 3195-3199.
17. England RW, Hardy KL, Kitajewski AM, Wong A, Kitajewski JK, Shawber CJ, et al. Propranolol promotes accelerated and dysregulated adipogenesis in hemangioma stem cells. Ann Plast Surg2014; 73: 119-124.
18. Li D, Li P, Guo Z, Wang H, Pan W. Downregulation of miR-382 by propranolol inhibits the progression of infantile hemangioma via the PTEN-mediated AKT/mTOR pathway. Int J Mol Med 2017; 39: 757-763.
19. Margileth AM, Museles M. Cutaneous hemangiomas in children. Diagnosis and conservative management. JAMA 1965; 194: 523-526.