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Research paper

# microRNA-143 acts as a suppressor of hemangioma growth by targeting Bcl-2

Chongqing Huang*, JingYong Huang, Pengyan Ma, Guanfeng Yu

*Department of Vascular Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou, China*

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ABSTRACT

Infantile hemangioma is the most common vascular tumor affecting infants, which is associated with clonal expansion of endothelial cells. The aim of this study is to determine the role of microRNA (miR)-143 in the growth and survival of hemangioma-derived endothelial cells (HemECs). We examined the expression of miR-143 in patients with proliferating-phase (n = 10) and involuting-phase (n = 8) hemangiomas. The effects of ectopic expression of miR-143 on the viability, proliferation, cell cycle distribution, and apoptosis of HemECs were explored. We also identified the target gene(s) that was involved in the activity of miR-143. It was found that proliferating hemangiomas had significantly ($P < 0.05$) lower levels of miR-143 than involuting counterparts. Reexpression of miR-143 significantly reduced the viability and proliferation of HemECs, while knockdown of miR-143 led to an increase in the proliferation of HemECs. Moreover, overexpression of miR-143 arrested HemECs at the G0/G1 phase and promoted caspase-3-dependent apoptosis. At the molecular level, miR-143 overexpression significantly promoted the expression of p21 and p53 and reduced the expression of cyclin D1, CDK2, CDK4, and Bcl-2. Silencing of Bcl-2 phenocopied the effect of miR-143 overexpression on the proliferation and apoptosis of HemECs. Furthermore, co-expression of Bcl-2 reversed the growth-suppressive effect of miR-143 on HemECs. Taken together, miR-143 acts as a suppressor in the growth of HemECs, at least partially, through downregulation of Bcl-2. Reexpression of miR-143 may represent a potential therapeutic strategy for the treatment of proliferating hemangiomas.

## 1. Introduction

Infantile hemangioma is the most common vascular tumor affecting infants, especially younger than 1 year of age (Castrén et al., 2016; Rouhana et al., 2015). These tumors are usually benign, but some can undergo malignant transformation (Gnarra et al., 2016). Typically, the life span of benign hemangiomas covers proliferating phase, involuting phase, and involuted phase (Takahashi et al., 1994). There is evidence that clonal expansion of endothelial cells carrying somatic mutations contributes to the development of hemangiomas (Boye et al., 2001; Walter et al., 2002). Vascular endothelial growth factor-A (VEGFA) signaling plays a pivotal role in regulating behaviors of hemangioma-derived endothelial cells (HemECs) (Marnat et al., 2015). Down-regulation of VEGF receptor-1 (VEGFR1) can augment VEGFA-dependent activation of VEGFR2, consequently facilitating HemEC growth (Jinnin et al., 2008). Pharmacological inhibition of VEGF signaling leads to growth suppression in HemECs (Pan et al., 2015). However, the mechanisms governing hemangioma growth is still not completely understood.

microRNAs (miRs) are small non-coding RNAs implicated in a broad range of physiological and pathological processes (Sun et al., 2015, 2016a, 2016b). They can negatively regulate gene expression via partial complementary binding to the 3′-untranslated region (UTR) of target mRNAs (Sun et al., 2016c, 2016d, 2016e). Several lines of evidence have linked dysregulation of miRs to progression of endothelial cell tumors (Gordillo et al., 2014; Nakashima et al., 2010). Impairment of miR synthesis secondary to knockdown of Dicer, a key enzyme that converts precursor to mature miR, can block endothelial cell tumor growth in a mouse model (Gordillo et al., 2014). Downregulation of miR-424 promotes the growth of senile hemangioma (Nakashima et al., 2010). miR-143 can modulate the biological behaviors of endothelial cells from different origins, e.g. inducing migration and angiogenesis in pulmonary arterial endothelial cells (Deng et al., 2015) and increasing the permeability of brain endothelial cells (Bai et al., 2016). This miR

*Abbreviations:* miR-143, microRNA-143; HemECs, hemangioma-derived endothelial cells; VEGFR1, VEGF receptor-1; UTR, untranslated region; EBM-2, Endothelial Basal Medium-2; FBS, fetal bovine serum; qRT-PCR, Quantitative real-time polymerase chain reaction; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; BrdU, 5-bromo-2′-deoxyuridine; PI, propidium iodide; RIPA, radioimmunoprecipitation assay; CDK, cyclin-dependent kinase; PARP, poly (ADP-ribose) polymerase

* Corresponding author at: Department of Vascular Surgery, The First Affiliated Hospital of Wenzhou Medical University, Wenzhou 325000, China.
*E-mail address:* christopherhuang@qq.com (C. Huang).

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acts as a tumor suppressor in multiple cancer types such as esophageal squamous cell carcinoma (He et al., 2016) and glioblastoma (Fu et al., 2016). Despite these studies, relatively little is known about the function of miR-143 in the growth of hemangioma.

In the present work, we aimed to determine the biological roles of miR-143 in HemEC proliferation, cell cycle progression, and apoptosis. Additionally, we also identified the direct target genes that mediated the activity of miR-143 in HemECs.

## 2. Materials and methods

### 2.1. Tissue samples

Proliferating- and involuting-phase hemangioma samples were surgically collected from 10 (8 females and 2 males; median age, 7 months) and 8 patients (7 females and 1 male; median age, 6 months), respectively. Each patient gave written informed consent for research purpose. All cases were confirmed by histological analysis at the Department of Vascular Surgery of The First Affiliated Hospital of Wenzhou Medical University (Wenzhou, China). This study protocol was approved by the Institutional Ethical Review Board of Wenzhou Medical University.

### 2.2. Measurement of miR-143 levels by quantitative real-time PCR (qRT-PCR) analysis

Detection of miR-143 expression was performed by qRT-PCR analysis, as described previously (Du et al., 2016). Briefly, total RNA was extracted from tissues and cells using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) and then reverse-transcribed into cDNA using the Taqman miRNA Reverse Transcription kit (Applied Biosystems, Carlsbad, CA, USA). Real-time PCR was carried out using the TaqMan miRNA assay system (Applied Biosystems). U6 small nuclear RNA was used as an endogenous control. The level of mature miR-143 was normalized to that of U6.

### 2.3. Isolation of HemECs

HemECs were isolated from a proliferating-phase infantile hemangioma, which was resected from a 6-month-old female patient, as described previously (Khan et al., 2006). HemECs were cultured in Endothelial Basal Medium-2 (EBM-2; Cambrex Bio Science, Walkersville, MD, USA) supplemented with 10% fetal bovine serum (FBS; Invitrogen Life Technologies). Confluent cells were subcultured using 0.05% trypsin-EDTA solution (Invitrogen Life Technologies). HemECs at passages 3–8 were used in this study.

### 2.4. miR-143 mimic, anti-miR-143 inhibitor, and plasmids

miR-143 mimic, anti-miR-143 inhibitor, and corresponding negative controls were purchased from QIAGEN (Hilden, Germany). Bcl-2-targeting shRNA and scrambled shRNA were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A plasmid expressing full-length open reading frame of *Bcl-2* (lacking the 3′-UTR) was purchased from Origene (Rockville, MD, USA).

### 2.5. Cell transfection

HemECs were transfected with miR-143 mimic, anti-miR-143 inhibitor, Bcl-2-targeting shRNA, and their negative controls (50 nM for each) using Lipofectamine 2000 (Invitrogen Life Technologies) as per the manufacturer's instructions. In some cases, HemECs were co-transfected with miR-143 mimic (50 nM) and the Bcl-2-expressing plasmid or empty vector (1 μg). For inhibitor experiments, cells were pretreated with Z-DEVD-FMK (20 μM; Calbiochem, San Diego, CA, USA), a specific caspase-3 inhibitor, 2 h before transfection with miR-143 mimic.

### 2.6. MTT assay

Cells were plated in triplicates in 96-well plates ($5 \times 10^3$ cells/well) and cultured for 48 or 72 h. Cell viability was measured by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were incubated with MTT (0.5 mg/ml; Sigma-Aldrich, St. Louis, MO, USA) for 4 h at 37 °C. The purple formazan crystals were dissolved in dimethyl sulfoxide, and absorbance was recorded at 570 nm.

### 2.7. BrdU incorporation assay

Cell proliferation was assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation assays. In brief, HemECs were seeded onto 96-well plates ($1 \times 10^4$ cells/well) and cultured for 48 h. BrdU (10 μM) was added to the wells and incubated for additional 4 h. The incorporation of BrdU into cellular DNA was detected using an anti-BrdU antibody. Absorbance of each well was measured at 450 nm. In some cases, HemECs were transfected with miR-143 mimic or control miR 24 h before treatment with recombinant VEGF (10 ng/ml; Peprotech, Rocky Hill, NJ, USA). After incubation for additional 24 h, cells were collected and subjected to BrdU incorporation assays.

### 2.8. Analysis of cell cycle and apoptosis by flow cytometry

For cell cycle analysis, cells were fixed in 70% ethanol at 4 °C overnight and stained with 50 mg/ml propidium iodide (PI; Sigma-Aldrich) for 30 min at 37 °C. DNA content was determined by flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA). For apoptosis detection, cells were incubated with annexin V-FITC (25 μg/ml; Beyotime Biotechnology, Hainen, China) and PI (25 μg/ml) for 10 min at room temperature in the dark. Stained cells were analyzed using a flow cytometer. Apoptotic cells were stained annexin V-FITC-positive and PI-negative.

### 2.9. Western blot analysis

Whole cellular extracts were prepared by lysing cells in radioimmunoprecipitation assay (RIPA) buffer (Beyotime, Haimen, China) containing the Complete Protease Inhibitor Cocktail (Roche, Indianapolis, IN, USA). Cytosolic fractions were prepared using the Mitochondria/Cytosol Fractionation Kit (BioVision, Mountain View, CA, USA), according to the manufacturer's protocol.

Protein samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred onto polyvinylidenedifluoride transfer membranes. The membranes were incubated overnight at 4 °C with the following primary antibodies: anti-p21, anti-p53, anti-cyclin D1, anti-cyclin-dependent kinase (CDK) 2, anti-CDK4, anti-cytochrome *c*, anti-cleaved caspase-3, anti-cleaved poly(ADP-ribose) polymerase (PARP), anti-Bcl-2, and anti-β-actin (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA). After washing, membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h. Immunoreactivity was detected by the chemiluminescence method (Thermo Scientific, Rockford, IL, USA). Protein signals were quantified using Quantity One software (Bio-Rad, Hercules, CA, USA).

### 2.10. Statistical analysis

Data are presented as mean ± standard deviation. Statistical differences were determined by the Student's *t*-test or one-way analysis of variance followed by Tukey's post-hoc test. $P < 0.05$ was considered statistically significant.