MicroRNA-424 suppresses the proliferation of hemangioma-derived endothelial cells by targeting VEGFR-2

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Received September 13, 2017; Accepted July 27, 2018

DOI: 10.3892/mmr.2018.9409

Abstract. Hemangioma (HA) is a type of benign tumor common in infancy. The main feature of HA is the abnormal proliferation of vascular endothelial cells. To date, the pathogenesis of HA remains unclear. Fully understanding the process of HA tumorigenesis is essential for developing novel treatment for HAs. Dysregulation of microRNAs (miRNAs/miR) has been reported to be involved in the development of various diseases, including HA. In the present study, the expression of miR-424 decreased in HA-derived endothelial cells (HemECs). To elucidate the role of miR-424 in HAs development, the present study overexpressed or inhibited miR-424 in HemECs, revealing that miR-424 overexpression significantly inhibited HemEC growth and promoted apoptosis, while the downregulation of miR-424 promoted cell growth and inhibited cell apoptosis. To elucidate the underlying mechanism, bioinformatic analyses were performed, the result of which demonstrated that the 3'-untranslated region of vascular endothelial growth factor receptor 2 (VEGFR-2) may be a target of miR-424. The result of a dual luciferase reporter assay confirmed that the expression of VEGFR-2 was inhibited by miR-424. In addition, it was revealed that the hyper-phosphorylation of protein kinase B (AKT) and extracellular signal-regulated kinase (ERK) in HemECs, and the restoration of miR-424 markedly inhibited the activation of AKT and ERK. In conclusion, these results indicated that miR-424 may target VEGFR-2 and inhibit HemECs growth, and that low expression of miR-424 in HemECs may lead to an increase in cell growth and a decrease in cell apoptosis. Thus, it was proposed that miR-424 may serve as a tumor suppressor in HemECs, and that VEGFR-2 may be a potential tumor suppressive target in HemECs and for the treatment of HA.

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Key words: microRNA-424, hemangioma, vascular endothelial growth factor receptor 2

Introduction

Hemangiomas (HAs) are benign neoplasms that proliferate rapidly, they are known to be the most common tumor to present in infancy (1). It is reported that 1/10 children will develop HAs, most of which are in the head or neck region (2). A previous research revealed that HAs is associated with low birth weight and premature infants (3). They examined 973 preterm infants and the results showed that the frequency of HAs is 12.7%, for the low-birth-weight infants below 1,000 g and below 1,500 g the rate was 15.6 and 22.9%, respectively. The development of HAs usually include 3 stages, an early proliferative phase (0-12 month), a plateau phase and an involution phase (1-8 years). Although HAs are benign, they required treatment because they might develop complications and resulted in mortality (4,5). To date, the molecular mechanisms underlying the occurrence and development of HAs are still unclear.

MicroRNAs (miRNAs) are a class of 19-24 nt small non-coding RNAs. It is clear that miRNAs negatively regulate mRNA translation by binding to the 3'-untranslated region (3' UTR) of target mRNAs. Since the first miRNA was identified in 1993, more and more miRNAs have been discovered; and nearly 1,500 miRNA sequences have reportedly been found (6). miRNAs play critical role in various biological processes, including cell growth (7), proliferation (8), differentiation (9) and apoptosis (10). During the last decade, a growing body of literature has confirmed that the dysregulation of miRNAs is involved in almost all diseases. A few studies have reported that miRNAs are aberrantly expressed in HAs. For example, Li *et al* (11) found that miR-382 is upregulated in infantile HA, and that the inhibition of miR-382 by propranolol prominently inhibits the progression of cells derived from infantile HA; A meta-analysis identified that miR-939, miR-9 and let-7 family are involved in regulating infantile HA (12); Venneti *et al* (13) found that miR-9 and miR-200a are dysregulated in HAs; And miR-143 was found to act as a suppressor of HA growth by

targeting Bcl-2 (14). These findings presented a new perspective for understanding the molecular mechanisms underlying the development of HAs.

miR-424 has an important role in regulating cell-autonomous angiogenesis (15), and studies have reported that downregulation of miR-424 contributes to the abnormal angiogenesis in senile HA (16). In this study, we examined the expression of miR-424, finding that miR-424 was reduced in HA-derived endothelial cells (HemECs), and demonstrating that miR-424 might be used to treat HAs, as the restoration of miR-424 levels in HemECs could effectively inhibit cell growth and induce apoptosis. Furthermore, we demonstrated that vascular endothelial growth factor receptor (VEGFR)-2, an important pro-angiogenic receptor, was a direct target of miR-424. VEGF-R2 was upregulated in HemECs and the overexpression of miR-424 clearly decreased VEGFR-2 and inhibited the activation of protein kinase B (AKT)/extracellular signal-regulated kinase (ERK) signaling cascades. Thus, our data demonstrated that miR-424 serves a critical role in HA pathogenesis and indicate that it may represent a therapeutic target in the treatment of HAs.

Materials and methods

Preparation of HA specimens. A case of proliferating-phase infantile HA specimen was collected from the Department of General Surgery, Affiliated with Xinhua Hospital (Shanghai, China). The tissue was washed with pre-cooled PBS (0˚C) and HemECs cells were isolated. The experimental protocol was approved by the Ethics Committee of Xinhua Hospital. Written informed consent was obtained from the parents of the patient.

Cell extraction, isolation and culture. Fresh HA sample was washed with PBS for three times, and minced with scissors. Then, 0.2% collagenase A was added and the mixture was maintained at 37˚C. After 1 h, the mixture was removed from the incubator and homogenized using glass homogenizers. Then the homogenate was filtered through a 40 μm cell strainer and the suspension was collected. HemECs were isolated from the suspension using CD31 immuno-magnetic beads and plated on cell culture plates in Endothelial Cell Medium (ScienCell Research Laboratories, Inc., San Diego, CA, USA). Human umbilical vein endothelial cells (HUVECs) used in the experiments were purchased from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China), and also cultured and maintained in Endothelial Cell Medium.

Cell transfection. Human miR-424 mimics, miR-424 inhibitors and the corresponding negative control miRNA (miR-Ctrl) were purchased from GenePharma (Shanghai, China). Cells were seeded onto 6-well plates ($2x10^5$ cells per well), 24 h later the cells were transfected with miR-424 mimics, miR-424 inhibitors or miR-Ctrl using Oligofectamine™ transfection reagent (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). For total RNA extraction TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used. For miRNA extraction, a mirPremier® microRNA Isolation kit (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) was used. The RNA was quantified by assessing the absorbance at 260 and 280 nm. Following this, reverse-transcription was performed using M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.). RT-qPCR was performed on ABI 7300 (Applied Biosystems, Foster, CA, USA) using the SYBR Green qPCR Master Mix (Tiangen, Shanghai, China). GAPDH mRNA or U6 snRNA was used as the endogenous control. All primers used in our study were as followed: VEGFR-2 forward, 5'-CACTGGTTGTACCTCAGCAC-3' and reverse, 5'-CGTACCAGAAGACACTTCGT-3'; GAPDH forward, 5'-GTGAACCATGAGAAGTATGACAA-3' and reverse, 5'-CATGAGTCCTTCCACGATAC-3 (GenePharma). The thermocycling conditions for qPCR were as follows: 10 min at 95˚C, followed by 40 cycles of 95˚C for 15 sec, 56˚C for 30 sec, and 70˚C for 30 sec. The 2-ΔΔCq method was used to analyze relative gene expression (17).

Construction of expression vectors. 3'UTR of VEGFR-2 was amplified using PCR and cloned into a psiCHECK-2 vector to generate the psiCHECK2-VEGFR-2-3'UTR (Luc-VEGFR-2-wt). The following primers were used to clone VEGFR-2 3'UTR forward, 5'-CTCGAGAAGGAAGCATCC ACACC-3' and reverse, 5'-GCGGCCGCGTTGTCGAAA TGAAAATC-3'. The PCR products were digested with Xho I and Not I and inserted into a psiCHECK vector. To generate the psiCHECK-VEGFR-2-mut-3'UTR (Luc-VEGFR-2-mut), a QuikChange mutagenesis kit (Stratagene; Agilent Technologies GmbH, Waldbronn, Germany) was used to perform site-directed mutagenesis of the miR-424 target sites in the VEGFR-2 3'UTR.

Cell proliferation assay. Cell proliferation was assessed using a Cell Counting kit-8 (Dojindo, Kumamoto, Japan), according to the manufacturer's instructions. 48 h after transfection with the indicated miRNAs, cell culture supernatants were discarded, and 10 μl CCK-8 solution was added, After 2 h, the absorbance values at 450 nm were measured. All the experiments were carried out in triplicate and repeated three times.

Dual luciferase reporter assay. For the dual luciferase reporter assay, HEK293T cells were seeded onto 96 well-plate ($5x10^3$ per well), After 24 h, the cells were co-transfected with pRL-TK (Promega, Madison, WI, USA), Luc-VEGFR2-wt or Luc-VEGFR2-mut and miR-424 or control mimics using lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). 24 h later dual-luciferase assay was performed according to specification (Promega). Luciferase activity was measures using a Victor Luminometer (Perkin Elmer, Waltham, MA, USA). The plasmid of pRL-TK containing Renilla luciferase was used as internal control. The experiments were carried out in triplicate and repeated 3 times.

Western blotting analysis. Cells were harvested and lysed in RIPA lysis buffer (Beyotime Institute of Biotechnology, Shanghai, China). Lysates were prepared from $1x10^7$ cells by dissolving the cell pellets in 100 μl lysis buffer. Lysates were then centrifuged at 12,000 rpm for 10 min, and supernatants were transferred to fresh Eppendorf tubes. The protein

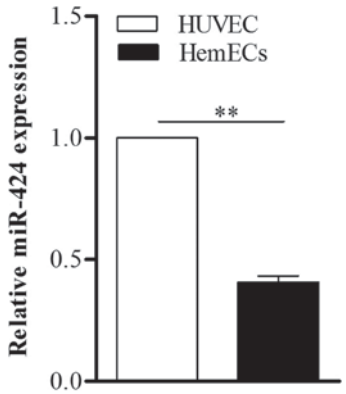

Figure 1. miR-424 expression is downregulated in HemECs. The expression of miR-424 was determined by reverse transcription-quantitative polymerase chain reaction in HemECs and HUVECs. **P<0.01, as indicated. HemECs, hemangioma-derived endothelial cells; HUVECs, human umbilical vein endothelial cells; miR, microRNA.

concentration was determined using a BCA protein assay kit (Beyotime Institute of Biotechnology). 30-50 μg protein was subjected to 10% SDS-PAGE and then electrophoretically transferred to a PVDF membrane (Millipore, Billerica, MA, USA). After blocking with blocking buffer (Beyotime Institute of Biotechnology) for 1 h at room temperature, the membrane was incubated with the indicated primary antibodies overnight at 4˚C. This was followed by incubation with HRP-conjugated secondary antibodies for 1 h. Positive signals were visualized using the ECL Advanced Solution (Bioworld, St. Louis, USA). Actin was used as the loading control. The antibodies used in this study were as follows: VEGFR-2 (1:2,000), ERK1/2 (1:2,000), p-ERK1/2 (1:2,000), p-AKT (1:2,000), AKT (1:2,000), and ACTIN (1:2,000). These antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA). All the experiments were repeated three times and the results are presented as the mean ± SEM.

Apoptosis analysis. After been treated as indicated, the cells were collected, washed and subjected to apoptosis analysis using an Annexin V-fluorescein isothiocyanate (FITC) kit (Beyotime Institute of Biotechnology), according to the manufacturer's instructions. Cells were analyzed using a FACScan flow cytometer with CellQuest software (BD Biosciences, Franklin Lakes, NJ, USA). Experiment was repeated three times and the results are presented as the mean ± SEM.

Statistical analysis. Differences between two groups were analyzed for statistical significance by using analysis of variance with a Tukey-Kramer post hoc test or the Student's t-test. Statistical analyses were conducted using Prism GraphPad software version 4 (GraphPad Software, Inc., La Jolla, CA, USA). Data are expressed as the mean ± standard error of the mean. P<0.05 was considered to indicate a statistically significant difference.

Results

miR-424 expression is downregulated in HemECs. To determine the role of miR-424 in HemECs, we first used qPCR to detect the expression of miR-424 in HemECs collected from tissue specimens obtained from a patient with proliferating-phase infantile HA. Compared to HUVECs, miR-424 was markedly low in HemECs (Fig. 1).

miR-424 overexpression inhibits cell proliferation and promotes cell apoptosis in HemECs. To further examine the possible function of miR-424 during HA development, miR-424 mimics or miR-ctrl were transfected into HemECs. After transfection with miR-424 mimics, the cells were collected and subjected to qPCR, the results of which showed that miR-424 was effectively upregulated in HemECs (Fig. 2A). The results of CCK-8 assay demonstrated that the upregulation of miR-424 resulted in decreased HemECs cell viability (Fig. 2B).

Additionally, we explored the possible role of miR-424 in HemECs apoptosis. miR-424 mimics or miR-ctrl were transfected into HemECs for 48 h, following which the cells were collected and subjected to an apoptosis assay. The results indicated that the overexpression of miR-424 in HemECs markedly promoted apoptosis (Fig. 2C).

VEGFR2 is a direct target of miR-424. Next, we explored how miR-424 exerted an inhibitory effect on HemECs proliferation, we used online miRNA target prediction databases (www.targetscan.org) to identify the targets of miR-424 in HemECs. The results showed that VEGFR-2 might be one such target (Fig. 3A). To further study whether VEGFR-2 was a direct target of miR-424, we performed a luciferase reporter gene assay. The wt-VEFGR2-3'UTR plasmid, mut-VEFGR2-3'UTR plasmid and miR-424/miR-Ctrl were co-transfected into HEK293T cells. As shown in Fig. 3B, compared with the cells co-transfected with miR-Ctrl, the luciferase activities of miR-424-transfected cells were suppressed by ~68%, while those cells co-transfected with the mut-VEFGR2-3'UTR plasmid did not exhibit altered luciferase activity. The aforementioned data indicate that miR-424 regulates VEGFR-2 expression through direct targeting the 3'UTR of VEGFR2.

miR-424 downregulates VEGFR2 expression in HemECs. VEGF is an endothelial cell mitogen and survival factor, involved in regulating vascular development during embryogenesis and blood-vessel formation in adults (18). VEGFR-2 is the most biologically important receptor for VEGF. Herein, we found that VEGFR-2 is a target of miR-424, following the induced overexpression of miR-424 in HemECs. HAs regulates endothelial cell migration, proliferation and survival. Here we found that VEGFR-2 was a target of miR-424, then we overexpressed miR-424 in HemECs, As shown in Fig. 4, compared with HemECs transfected with miR-Ctrl, VEGFR-2 mRNA levels were significantly suppressed in those HemECs transfected with miR-424 mimics (Fig. 4A); and the overexpression of miR-424 significantly decreased the expression of VEGFR2 in HemECs (Fig. 4B). The results demonstrated that high expression of VEGFR-2 in HemECs might be attributed to miR-424 downregulation. These results further verified that VEGFR2 is a bona fide target of miR-424.

Inhibition of VEGFR2 is responsible for the suppressive effects of miR-424 in HemECs. To explore whether miR-424 inhibited HemECs growth is mediated by VEGFR-2, we

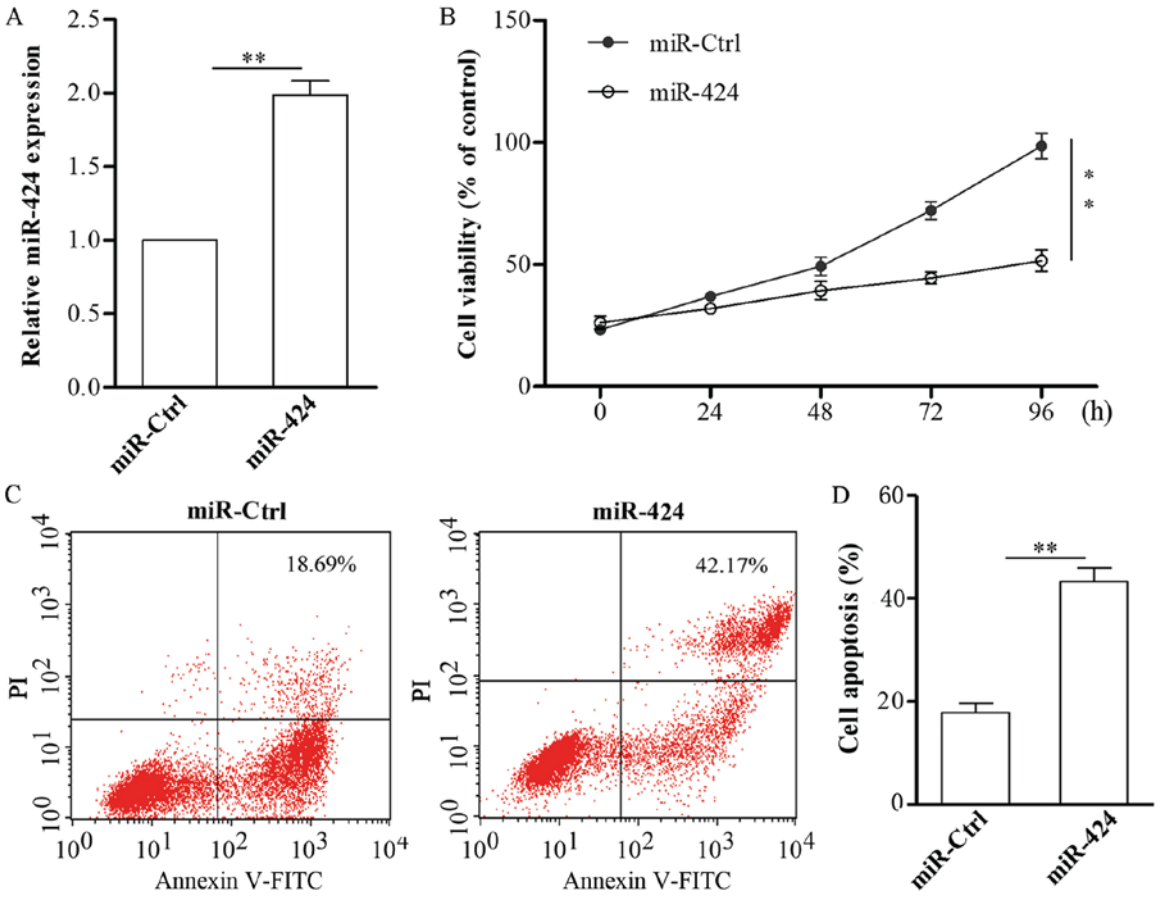

Figure 2. Overexpression of miR-424 inhibits cell proliferation and promotes cell apoptosis in HemECs. (A) The expression of miR-424 in HemECs was detected by reverse transcription-quantitative polymerase chain reaction. (B) HemECs proliferation was detected via Cell Counting kit-8 assay. (C) Apoptosis of HemECs was analyzed using a FITC/Annexin V Apoptosis Detection kit, following transfection with miR-424 or miR-Ctrl. (D) Quantification of flow cytometry. **P<0.01, as indicated. HemECs, hemangioma-derived endothelial cells; HUVECs, human umbilical vein endothelial cells; miR, microRNA; FITC, fluorescein isothiocyanate; PI, propidium iodide; Ctrl, control.

induced VEGFR-2 knockdown in HemECs using RNAi. The results showed that, following the transfection of HemECs with VEGFR2 siRNA, VEGFR2 mRNA and protein expression was effectively inhibited (Fig. 5A and B). Results from the CCK-8 assay showed that HemECs growth was inhibited by the suppression of VEGFR-2 (Fig. 5C). These data demonstrated that miR-424 suppresses the proliferation of HemECs via targeting VEGFR-2.

miR-424 inhibits the phosphorylation of AKT and ERK in HemECs. To determine whether miR-424 suppresses the downstream signaling of VEGFR-2 in HemECs, we transfected miR-424 mimics or miR-Ctrl into HemECs and then detected the expression of AKT, p-AKT, ERK1/2 and p-ERK1/2 via western blotting. As shown in Fig. 6, the upregulation of miR-424 clearly inhibited phosphorylation of AKT and ERK1/2. These data reveal that overexpression of miR-424 suppresses HemECs growth partly via regulation of the AKT and ERK signaling pathways.

Discussion

miRNAs are a group of endogenously expressed small non-coding RNAs that negatively regulate protein expression by targeting the 3' UTR of target mRNAs. It was reported

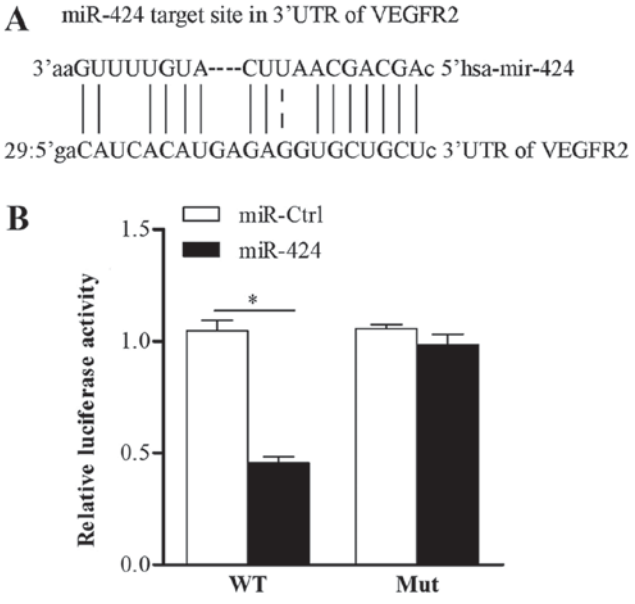

Figure 3. VEGFR2 is a direct target of miR-424. (A) VEGFR-2 may be a target of miR-424. (B) 293T cells were co-transfected with Luc-VEGFR-2-wt or Luc-VEGFR-2-mut, together with pRL-TK, control miRNA or miR-424 mimics, as indicated. Following 48 h, firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. *P<0.05, as indicated. VEGFR2, vascular endothelial growth factor receptor 2; miR, microRNA; Luc, luciferase; WT, wild type; Mut, mutant; Ctrl, control.

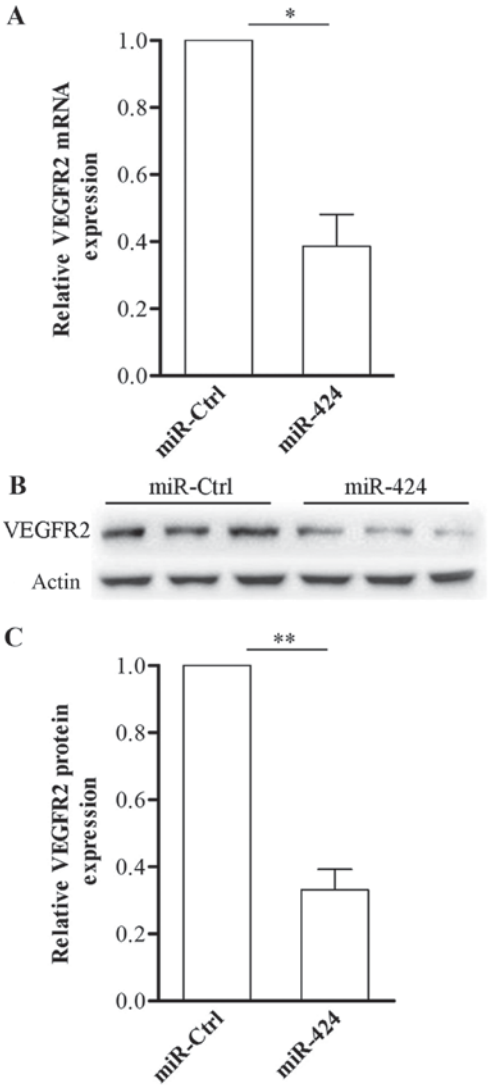

Figure 4. miR-424 downregulates VEGFR2 expression in HemECs. (A) HemECs were transfected with control miRNA or miR-424 mimics. Following 48 h, cells were collected and reverse transcription-quantitative polymerase chain reaction was performed to examine VEGFR2 mRNA levels. (B and C) HemECs were transfected with control miRNA or miR-424 mimics. Following 48 h, VEGFR2 was detected via western blotting. β-actin was used as the internal control. *P<0.05 and **P<0.01, as indicated. VEGFR2, vascular endothelial growth factor receptor 2; miR/miRNA, microRNA; HemECs, hemangioma-derived endothelial cells; Ctrl, control.

that 30% of the protein-coding genes in human are regulated by miRNAs (19,20). There have been increasing number of reports describing how the t dysregulation of miRNAs contributes to tumorigenesis and tumor development (21,22). Herein, we evaluated the downregulation of miR-424 in HemECs, determining that overexpressed miR-424 in HemECs markedly inhibited cell growth and promoted apoptosis. Further experiments demonstrated that VEGFR-2 is a direct target of miR-424, and that miR-424 inhibits HemECs development through VEGFR-2-mediated ERK signaling pathway. These findings indicate that miR-424 could represent a therapeutic target for treatment of HA.

miR-424 has been identified as aberrant in various tumor types, and many researchers have declared that miR-424 exerts tumor suppressor role (16,23). For example, Yu *et al* (24) found that miR-424 is downregulated in hepatocellular carcinoma, and that the overexpression of miR-424 serves a tumor suppressor role through targeting proto-oncogene c-Myb. In chronic lymphocytic leukemia, the ectopic expression of miR-424 resulted in significantly decreased expression of the oncogene PLAG1 (25,26). It was also reported that miR-424 in cervical cancer cells suppressed cell growth, migration and invasion (27). Concordant with these discoveries, miR-424 was found to be dysregulation in senile HA (16). Studies have stated that low miR-424 expression levels contribute to the upregulation of MEK1 or cyclin E1 in senile HA, which may then cause abnormal tumor cell proliferation.

Yang *et al* (28) revealed that miR-424 inhibit cell proliferation, migration and tube formation capabilities and the development of infantile skin HA. They further demonstrated that miR-424 explore the role through suppressing the bFGF/FGFR1/ERK1/2 pathway. Here we also found that the expression of miR-424 was lowed in HemECs, and that the overexpression of miR-424 in HemECs clearly inhibited HemECs growth and induced apoptosis. To elucidate the molecular mechanism underlying the growth suppressive role of miR-424 in HemECs, we predicted the possible targets of miR-424 using bioinformatics analysis and verified that VEGFR-2 is a direct target of miR-424 in HemECs. Transfection of miR-424 into HemECs resulted in significantly decreased VEGFR-2 expression; miR-424 also significantly inhibited VEGFR2 3' UTR luciferase reporter activity, while showing no effect on the mut-VEGFR-3' UTR reporter activity. These data demonstrate that miR-424 may suppress HA development partly through reducing VEGFR-2 expression and this is differs from Yang (28) and her colleagues' report, which means miR-424 exert inhibition role in HA via many ways.

Angiogenesis is considered as a critical event in tumor progression (29,30). Currently, the inhibition of angiogenesis is a strategy widely proposed for treatment of various diseases, particularly malignancies (31-34). Angiogenesis is a physiological process that involves multiple cellular processes, including endothelial cell proliferation, migration, and morphological differentiation, and is regulated by various growth factors and intracellular signaling pathways (35). VEGFs serve key roles in regulating angiogenesis. VEGFR-2 is a major receptor in the VEGF signaling pathway, and regulates cell migration, proliferation, and angiogenesis (36). It is well known that HAs are tumors formed by hyper-proliferation of vascular endothelial cells, which is caused by elevated VEGF signaling transduction through VEGFR-2. In our previous study, we demonstrated that increased VEGFR-2 expression is involved in the development of primary HemECs, though the underlying mechanisms remain unknown. The results of our investigation revealed that VEGFR-2 is a direct target of miR-424, and that miR-424 can negatively regulate the expression of VEGFR-2; these discoveries may indicate novel mechanism of post-transcriptional control of VEGFR-2.

In our study, we also examined whether miR-424 affects downstream signaling pathway of VEGFR-2. The results demonstrated that overexpressed miR-424 in HemECs inhibited phosphorylation of AKT and ERK1/2. These data demonstrate that miR-424 inhibits HemECs progression by targeting VEGFR-2 through the AKT/ERK signaling pathway.

In conclusion, our study suggest that the downregulation of miR-424 contributes to HA development. Our data also

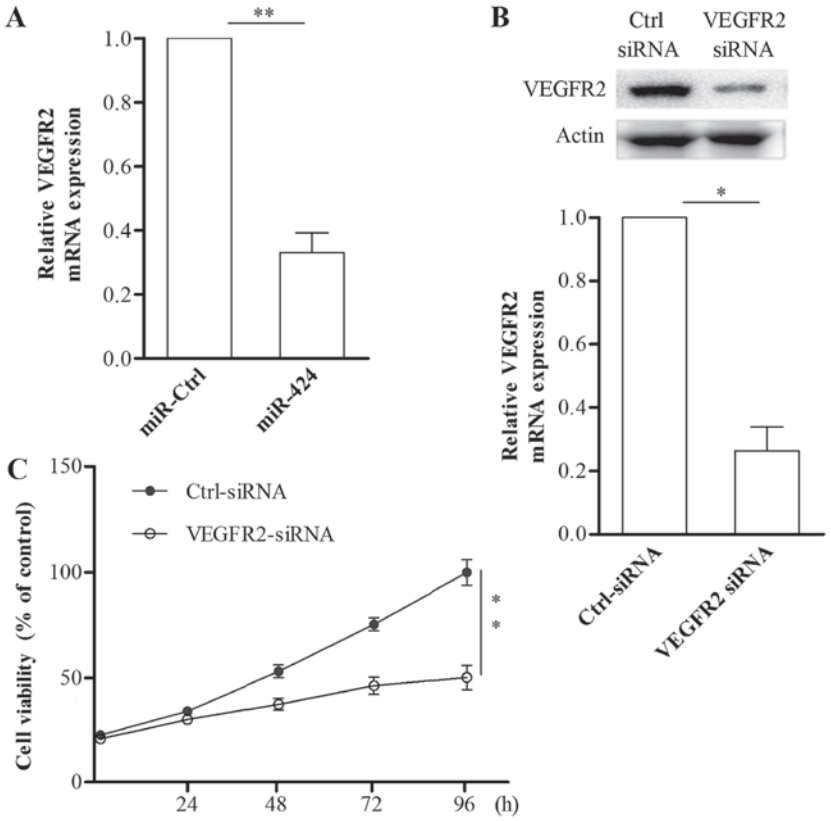

Figure 5. Inhibition of VEGFR-2 is responsible for the suppressive effect of miR-424 in HemECs. Following transfection with VEGFR-2 siRNA, the VEGFR-2 (A) mRNA and (B) protein levels in HemECs were detected via reverse transcription-quantitative polymerase chain reaction and western blotting, respectively. (C) Determination of HemECs proliferation with a Cell Counting kit-8 assay following transfection with VEGFR-2 siRNA or Ctrl-siRNA. *P<0.05 and **P<0.01, as indicated. VEGFR2, vascular endothelial growth factor receptor 2; miR, microRNA; siRNA, small interfering RNA; HemECs, hemangioma-derived endothelial cells; Ctrl, control.

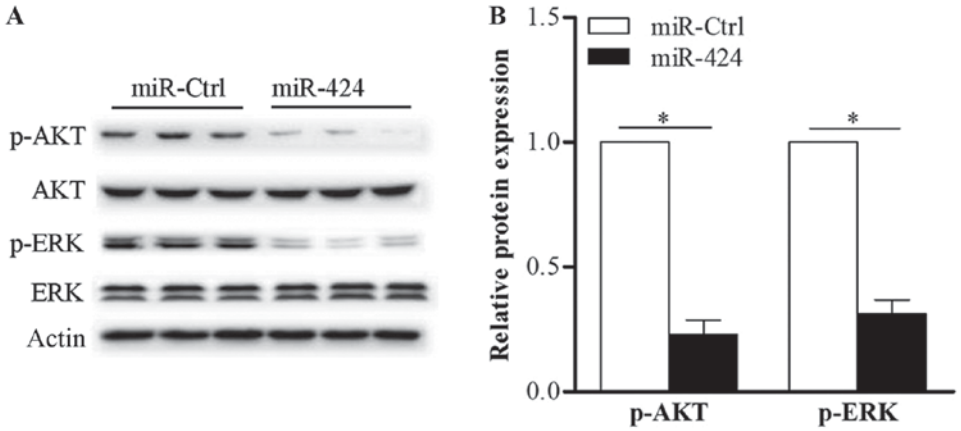

Figure 6. miR-424 inhibits the AKT and ERK signaling pathways in HemECs. (A) Following the transfection of miR-424 mimics or miR-ctrl into HemECs, the expression of AKT, p-AKT, ERK1/2 and p-ERK1/2 was determined via western blotting. (B) Upregulation of miR-424 inhibited the phosphorylation of AKT and ERK in HemECs. Actin was used as an internal control. *P<0.05, as indicated. miR, microRNA; siRNA, small interfering RNA; HemECs, hemangioma-derived endothelial cells; Ctrl, control; AKT, protein kinase B; ERK, extracellular signal-regulated kinase; p-, phosphorylated.

indicate that miR-424 is clearly downregulated in HemECs, and that the restoration of miR-424 inhibits HemECs growth and induced apoptosis partly through suppressing the VEGFR-2 pathway. These results help us to understand the molecular mechanisms of HA development and allow us to propose miR-424 as a potential biomarker and therapeutic target for HA. While, all data above is based on only one patient, this is a shortage of the study.

Acknowledgements

Not applicable.

Funding

The present study was supported by by the National Natural Science Foundation of China (grant no. 81572673), the Science

and Technology Commission Foundation of Shanghai, China (grant no. 13140903802), the Medicine and Engineering Cross Foundation of Shanghai Jiaotong University (grant no. YG2012MS33) and Shanghai Science and Technology Department (grant no. 15140901600).

Availability of data and materials

The datasets used or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

ZF and JO wrote the manuscript. ZQ and YL treated the patient and collected the clinical samples. ZF, MQ, XQ, YD, SW, ZQ and YL performed the experiments. ZF, MQ, XQ and JO contributed to study design, data analysis and interpretation. All authors reviewed and approved the manuscript.

Ethics approval and consent to participate

The experimental protocol was approved by the Ethics Committee of Xinhua Hospital. Written informed consent was obtained from the parents of the patient.

Patient consent for publication

Written informed consent was obtained for publication.

Competing interests

The authors declare that they have no competing interests.

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