Original Research

MiR-200c-3p increased HDMEC proliferation through the notch signaling pathway

Xianyu Hu[1,*], Suwen Bai[2,*], Lingyi Li[3,*], Pengfei Tian[2], Sun Wang[3], Ning Zhang[2], Bing Shen[2], Juan Du[2,4] and Shengxiu Liu[3]

[1]Department of General Surgery, The First Affiliated Hospital of Anhui Medical University Hefei, Anhui 230022, China; [2]School of Basic Medical Sciences, Anhui Medical University, Hefei, Anhui 230032, China; [3]Department of Dermatology, The First Affiliated Hospital of Anhui Medical University, Hefei, Anhui 230022, China; [4]Longgang District People's Hospital of Shenzhen & The Third Affiliated Hospital (Provisional) of The Chinese University of Hong Kong, Shenzhen, Guangdong 518172, China

Corresponding author: Shengxiu Liu. Email: liushxiu@126.com; Juan Du. Email: dujuan@chk.edu.cn

*These authors contributed equally to the work.

Impact statement

Hemangiomas mainly occur in the head and neck areas, often leading to psychological problems, even life-threatening. In the current study, we first mined out the potential biomarker miR-200c-3p based on the bioinformatic analysis in GSE69136, and after merging with another RNA-seq dataset GSE43742, focused on the downstream of Notch signaling pathway. The impaction of miR-200c-3p on the proliferation of hemangiomas cells was validated in by CCK8, and the marker Ki67, PCNA. What's more, we used ELISA to detect the expression of γ-secretase, with or without inhibiting or Notch signaling by DAPT. All in all, this study illustrates the downregulation of miR-200-3p increased HDMEC proliferation through the Notch signaling pathway, which might be a promising treatment target in the future.

Abstract

Excessive proliferation of vascular endothelial cells can cause hemangioma. Although typically benign, hemangiomas can become life-threatening. The microRNA miR-200c-3p is abnormally expressed in some types of tumors, but its expression, biological role, and mechanism of action in infantile hemangioma remain to be fully elucidated. The expression levels of miR-200c-3p in hemangioma tissue were compared with those in adjacent healthy tissue by using bioinformatics analyses and TargetScan. Western blot, enzyme-linked immunosorbent assay, and Cell Counting Kit 8 analyses were used to determine the biological function and site of action of miR-200c-3p in human dermal microvascular endothelial cells (HDMECs). MiR-200c-3p was one of the top 10 differentially expressed genes between healthy tissue, and hemangiomas tissues, having markedly decreased expression in hemangioma tissue. Reduction of miR-200c-3p expression in HDMECs through the transfection of a miR-200c-3p inhibitor significantly increased HDMEC proliferation. The addition of the Notch signaling pathway inhibitor DAPT to HDMECs transfected with the miR-200c-3p inhibitor eliminated the inhibitor-induced enhancement of proliferation in HDMECs. These findings indicate that miR-200c-3p targets the Notch signaling pathway to promote the proliferation of vascular endothelial cells, suggesting that miR-200c-3p plays an important role in the pathogenesis of hemangioma.

Keywords: Infantile hemangioma, miR-200c-3p, notch signaling pathway, cell proliferation

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Introduction

Hemangioma, known as infantile hemangioma in infants and newborns, is a congenital, benign tumor or vascular malformation that develops in the skin and soft tissue. The incidence of infantile hemangioma is 5% to 10% in newborns, and its clinical features include a proliferating phase, an involuting phase, and an involuted phase.[1] The onset of infantile hemangioma often occurs three to six months after birth and is rapidly followed by the proliferative phase.[2] In the proliferative phase, blood flow to the hemangioma markedly increases, resulting in a rapid increase in tumor volume and the suppression of symptoms in surrounding tissues and organs.[3] In the regression phase, the tumor does not grow but naturally degenerates, and in most children, the skin where the tumor formerly was shows no obvious differences from surrounding skin. Although hemangiomas are often benign, 60% of them occur in the head and neck area, affecting appearance, reducing quality of life, and potentially causing

psychological problems. In addition, the occurrence of hemangiomas in certain areas can cause serious complications, such as interference with vision.[4] The current treatment options for hemangioma (including chemotherapy or surgery) are insufficient to fully treat hemangioma.[5,6]

HDMECs are among the most common cell types used to investigate the mechanisms of hemangioma pathogenesis. Studying HDMECs, Wang *et al.*[7] found that rapamycin inhibited the proliferation of hemangioma cells through the mTOR-FABP4 pathway. Nakashima *et al.*[8] focused on the abnormal angiogenesis caused by miR-424 via MEK1/cyclin E1 in senile hemangioma by using HDMECs. Mechanistic studies have shown that the Notch signaling pathway plays a key role in the development and stabilization of blood vessels. The pathogenesis of infantile hemangioma involves three Notch receptors (Notch-1, Notch-3, and Notch-4) and four effectors, Hairy Enhancer of split (Hes), Hes-related with YRPW motif 1 and 2 (HEY1 and HEY2), and Hey-like.[9] The transcript levels of Notch-1, Notch-3, Notch-4, Jagged-1, and delta-like ligand 4 are all higher in infantile hemangioma than in the placenta (a tissue commonly used for comparison).[10–13] In contrast, the levels of Notch-2 are significantly reduced in both proliferative and regressive infantile hemangioma.[14]

MicroRNA (miRNA) is an evolutionarily highly conserved class of non-coding, small, single-stranded RNA (generally 18–22 nucleotides in length). MiRNAs are involved in the regulation of various pathophysiological processes, such as cell proliferation, differentiation, and apoptosis.[15,16] MiRNAs can regulate the expression of many genes simultaneously and typically affect signaling pathways at several levels. They are widely involved in the regulation of endothelial cell functions and play important roles in stabilizing these functions.[1,17,18] A member of the miR-200 family, miR-200c-3p, is located on chromosome 12. Low expression of miR-200c-3p in some types of cancer, such as poorly differentiated breast cancer, glioblastoma, clear cell kidney cancer, and colon cancer, leads to enhanced cancer aggressiveness.[19–22] However, the biological function of miR-200c-3p in infantile hemangioma has not been determined.

The aim of the present study was to investigate the expression level and role of miR-200c-3p in infantile hemangioma and the underlying mechanism. We also explored the specific effects of miR-200c-3p and the Notch signaling pathway on infantile hemangioma to gain insights into the molecular mechanisms underlying infantile hemangioma.

Materials and methods

Identification of differentially expressed genes

Two gene expression profiling data sets (GSE43742 and GSE69136) were obtained from the GEO database. GSE43742 contains data on human dermal microvascular endothelial cells (HDMECs) and human endometrial microvascular endothelial cells.[23] The GSE69136 profile includes data from12 surgical specimens from children with infantile hemangioma and 4 specimens of adjacent skin that appeared healthy.[24] The differentially expressed genes (DEGs) were determined using the GEO database through a GEO2R analysis (http://www.ncbi.nlm.nih.gov/geo/geo2r/) based on a cutoff of the absolute log2-fold change (|log2FC|) greater than 1.0 and $P < 0.05$.

DEG gene ontology and pathway enrichment analysis

The miR-200c-3p target genes were predicted using the online database TargetScan. To obtain the DEGs of interest, we used Venn diagram analysis to determine the miR-200c-3p target genes that intersected with the DEGs in the HDMEC sequencing data (GSE43742) (cutoff, $P < 0.05$, $|\log2FC| > 1.0$). The Database for Annotation, Visualization, and Integrated Discovery (http://david.abcc.ncifcrf.gov/) online tool was used for enrichment analysis and to visualize the Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway map of the DEGs. The cutoff criterion was $P < 0.01$.

Cell culture and transfection

The HDMEC cell line was acquired from the Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in serum-free Dulbecco's modified Eagle medium (DMEM, Gibco BRL, Grand Island, NJ, USA) containing 10% fetal bovine serum (Gibco BRL) at 37°C. The cells (4×10^5) were maintained in 6-well plates overnight. The cells were then transfected with miR-200c-3p mimics (5′-UAAUACUGCCGGGUAAUGAUGGA-3′) or miR-200c-3p inhibitor small interfering RNA (5′-UCCAUCAUUACCCGGCAGUAUUA-3′) at a concentration of 5 nM by using Lipofectamine 3000 (Invitrogen, Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The cell transfection efficiency was determined 24 h after transfection, and the cells were used for experiments.

Western blot assay

The HDMECs were harvested following various treatments and extracted using lysis buffer (in mM: 20 Tris, 150 NaCl, 1 EDTA, and 1 EGTA; 1% Triton X-100; 0.1% sodium dodecyl sulfate, and 1% protease inhibitor cocktail; pH 7.4). Equal amounts of cell extracts were separated on 12% sodium dodecyl sulfate–polyacrylamide gel electrophoresis gels. Separated protein bands were transferred into polyvinylidene fluoride membranes (EMD Millipore Corporation, Billerica, MA, USA), which were blocked in 5% skim milk made from powder. Primary antibodies included against CD31 (Affinity Biosciences, OH, USA); Ki67 (Affinity Biosciences, OH, USA); proliferation cell nuclear antigen (PCNA) (Affinity Biosciences, OH, USA), a marker commonly used to indicate cell proliferation; and β-tubulin (Affinity Biosciences, OH, USA) were diluted according to the manufacturer's instructions, added, and the membranes were incubated overnight at 4°C. Then, horseradish peroxidase-linked secondary antibodies (Abcam, Cambridge, MA, USA) were added at 1:1000 dilution and the membranes were incubated at room temperature for 2 h. The membranes were then washed with phosphate-buffered saline (Gibco BRL) and the bands

were visualized using an ECL-Plus/kit (GE Healthcare, Piscataway, NJ, USA) according to the kit's manufacturer's instructions.

Cell counting kit 8 assay

HDMECs were seeded in 96-well plates at a density of 2000 cells per well. Six replicates were established for each group. Each well contained 100 mL of DMEM with 10% fetal bovine serum and 10 mL of Cell Counting Kit 8 (CCK-8; Dojindo Laboratories, Kumamoto, Japan) solution. The absorbance value of each well at 450 nm was measured after the cells were cultured for 2 h at 37°C.

qPCR

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). One microgram of total RNA was reverse transcribed into cDNA using a reverse transcription kit (Thermo, Waltham, Massachusetts, USA), and quantitative PCR (qPCR) was performed on a Bio-Rad CFX system using the SYBR Green I qPCR kit (TAKARA, Dalian, China). GAPDH was used as an internal reference

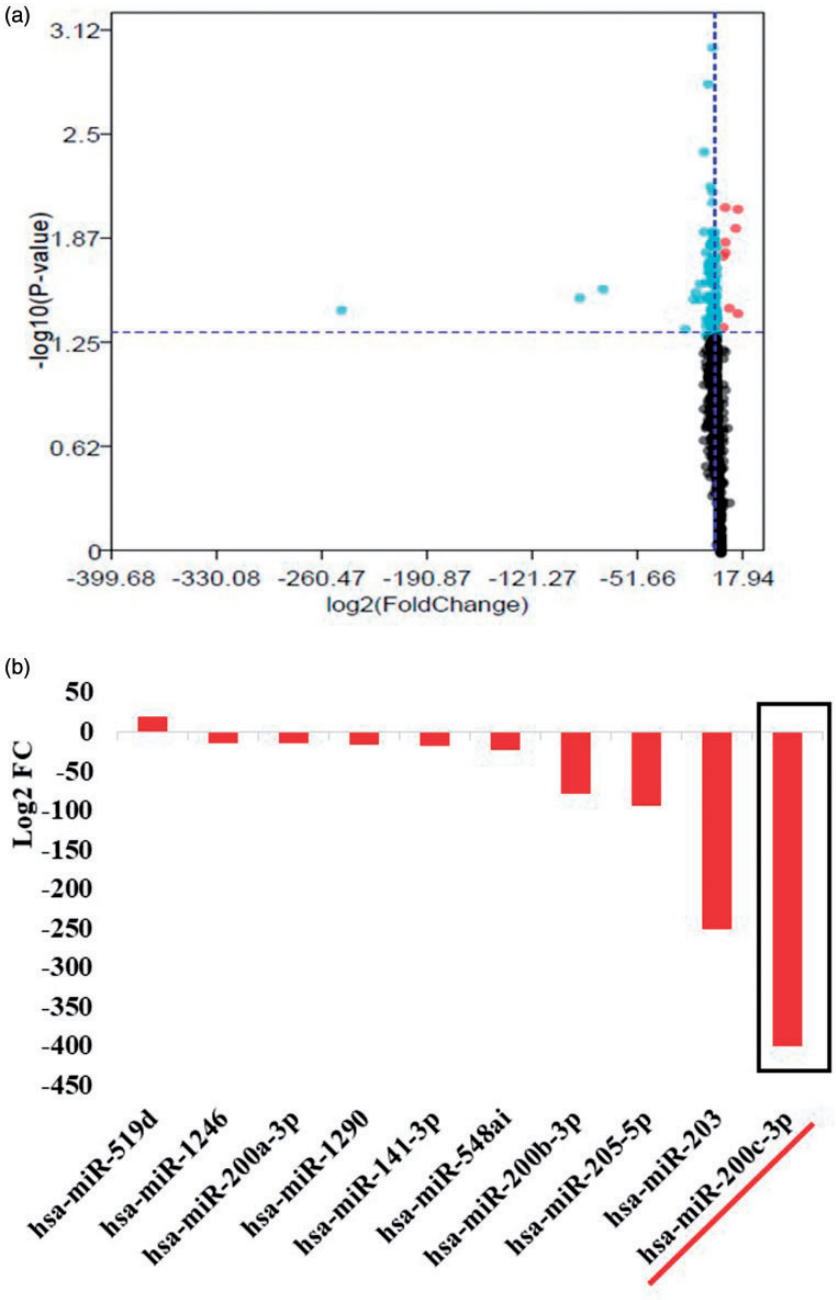

Figure 1. miR-200c-3p is significantly underexpressed in hemangioma. (a) The GSE69136 data set was analyzed by GEO, and the scatter plot shows miRNAs with the greatest log2 fold change (Log2FC) values. (b) The histogram indicates the 10 miRNAs with the greatest log2FC changes. (A color version of this figure is available in the online journal.)

gene to normalize RNA levels between different samples for comparison of transcription levels. All the gene expression data were normalized to the expression of the reference gene 36B4. Data were analyzed using the ΔΔCt method with GAPDH as the constitutive marker. The specific primers used are listed in Supplemental material Table S1.

Statistical analysis

To detect differences among treatments, one-way analysis of variance was used. Paired Student's *t*-tests were used to evaluate the statistical significance of differences between two groups. Differences detected in two-sided tests with $P < 0.05$ were considered statistically significant.

Results

miR-200c-3p is downregulated in infantile hemangioma tissue

After downloading the GSE69136 data set from the GEO database and performing a quality inspection of the original microarray data, we excluded the data corresponding to 8 specimens obtained from patients with lymphatic malformation but retained those of 12 surgical specimens obtained from children with infantile hemangioma and 4 specimens of adjacent skin that appeared healthy. The remaining data in the GSE69136 data set were then analyzed using GEO2R to detect genes differentially expressed between healthy tissue and infantile hemangioma tissue (Figure 1(a)). We detected 128 significantly downregulated DEGs and 11 significantly upregulated DEGs, both of which were used for subsequent bioinformatic analysis. The expression levels of the top ten downregulated DEGs are shown in Figure 1 (b). Among them, miR-200c-3p was markedly downregulated in the specimens from children with infantile hemangioma.

Enrichment of miR-200c-3p target genes in the notch signaling pathway

We identified 1190 miR-200c-3p target genes predicted by TargetScan. The intersection of these target genes

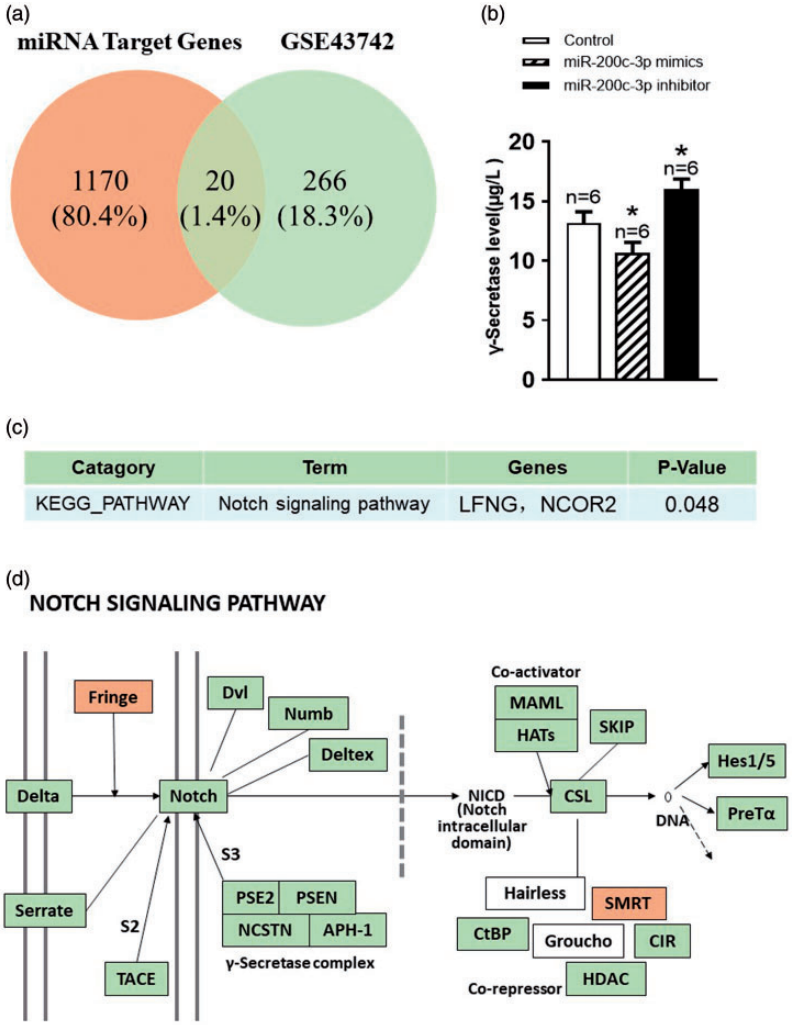

Figure 2. Enrichment of miR-200c-3p target genes in the Notch signaling pathway. (a) Venn diagram showing that 20 genes are in both the miR-200c-3p target gene group and the differentially expressed genes in the GSE43742 data set. Percentages are of the total number of genes (1456) in the analysis. (b) Expression levels of γ-secretase detected in cell supernatants using an ELISA kit. Data are presented as the means ± SD from six independent experiments; *$P < 0.05$. (c) Kyoto Encyclopedia of Genes and Genomes (KEGG) signaling pathway analysis results and (d) Notching signaling pathway diagram. (A color version of this figure is available in the online journal.)

with the DEGs in the GSE43742 data set identified 20 DEGs as target genes of miR-200c-3p (Figure 2(a)). Most of these genes produce proteins with functions related to endothelial cell proliferation. Among those 20 DEGs, LFNG and NCOR2 (which encodes the nuclear receptor corepressor 2 protein) are involved in the Notch signaling pathway, which mediates endothelial cell proliferation in hemangioma (Figure 2(c) and (d)). We verified the association of miR-200c-3p with LFNG and NCOR2 using Western blot and qPCR. The expression of LFNG and NCOR2 was decreased in cells transfected with miR-200c-3p mimics but increased in cells transfected with miR-200c-3p inhibitor (Figure 4(d), (f), and (g)). The intramembrane protease γ-secretase is a key marker of Notch signaling pathway activity. Thus, we measured the effect of overexpressing or knocking down miR-200c-3p in HDMECs through transfection with miR-200c-3p mimics or inhibitor, respectively, on the levels of γ-secretase by using an enzyme-linked immunosorbent assay (ELISA). The results of this analysis were consistent with those of our bioinformatics analysis. As shown in Figure 2(b), the levels of γ-secretase were significantly decreased in cells transfected with miR-200c-3p mimics but significantly increased in cells transfected with miR-200c-3p inhibitor, suggesting that miR-200c-3p is associated with the Notch signaling pathway.

Inhibition of the notch signaling pathway decreases HDMEC proliferation

The Notch signaling system plays an important role in angiogenesis in infantile hemangioma, and is strongly activated in infantile hemangioma tissue.[10,11,14,25] Consistent with these observations, the expression level of γ-secretase in HDMECs was significantly decreased by treatment with an inhibitor of the Notch signaling pathway (DAPT, 200 μM) (Figure 3(a) and (b)). Vascular endothelial growth factor (VEGFR) and the endothelial adhesion receptor CD31 are known to be involved in HDMEC proliferation.[9] Thus, we treated HDMECs with 200 μM of DAPT to verify the function of Notch signaling in HDMEC proliferation. The results of our Western blot analysis revealed that DAPT significantly decreased CD31 (Figure 3(c) and (d)) and VEGFR expression levels (Figure 3(c) and (e)). In addition, the CCK-8 assay revealed that treatment with DAPT significantly inhibited HDMEC proliferation (Figure 3(f)). Taken together, these findings indicated that inhibition of the Notch signaling pathway decreased HDMEC proliferation.

MiR-200c-3p affects the proliferation of HDMECs through inhibition of the notch signaling pathway

Because miR-200c-3p was markedly downregulated in the hemangioma specimens, we hypothesized that it might

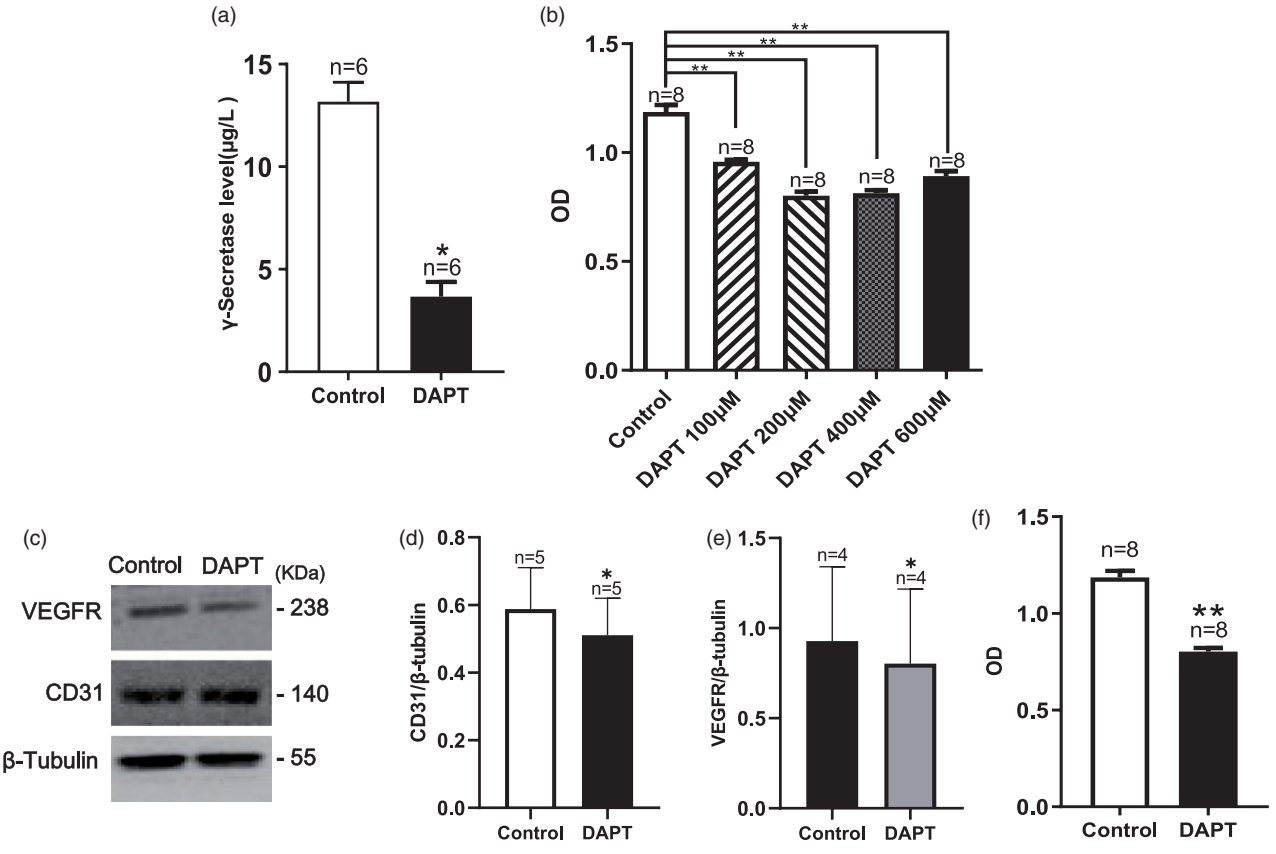

Figure 3. Inhibiting the Notch signaling pathway decreases HDMEC proliferation. (a) Expression levels of γ-secretase in supernatants from human dermal microvascular endothelial cells (HDMECs) treated with DAPT or the vehicle control (DMSO) as measured by an ELISA. (b) HDMECs treated with increasing concentrations of DAPT to find the optimal inhibitory concentration for WESTERN blot analysis. Inhibiting the Notch signaling pathway significantly decreased the protein expression levels of CD31 (c and d) and VEGFR (c and e). (f) Results of CCK-8 analyses indicating that DAPT inhibits HDMEC proliferation. OD represents optical density. Data are presented as the means ± SD from the number of independent experiments indicated above each bar; $^{*}P < 0.05$, $^{**}P < 0.01$.

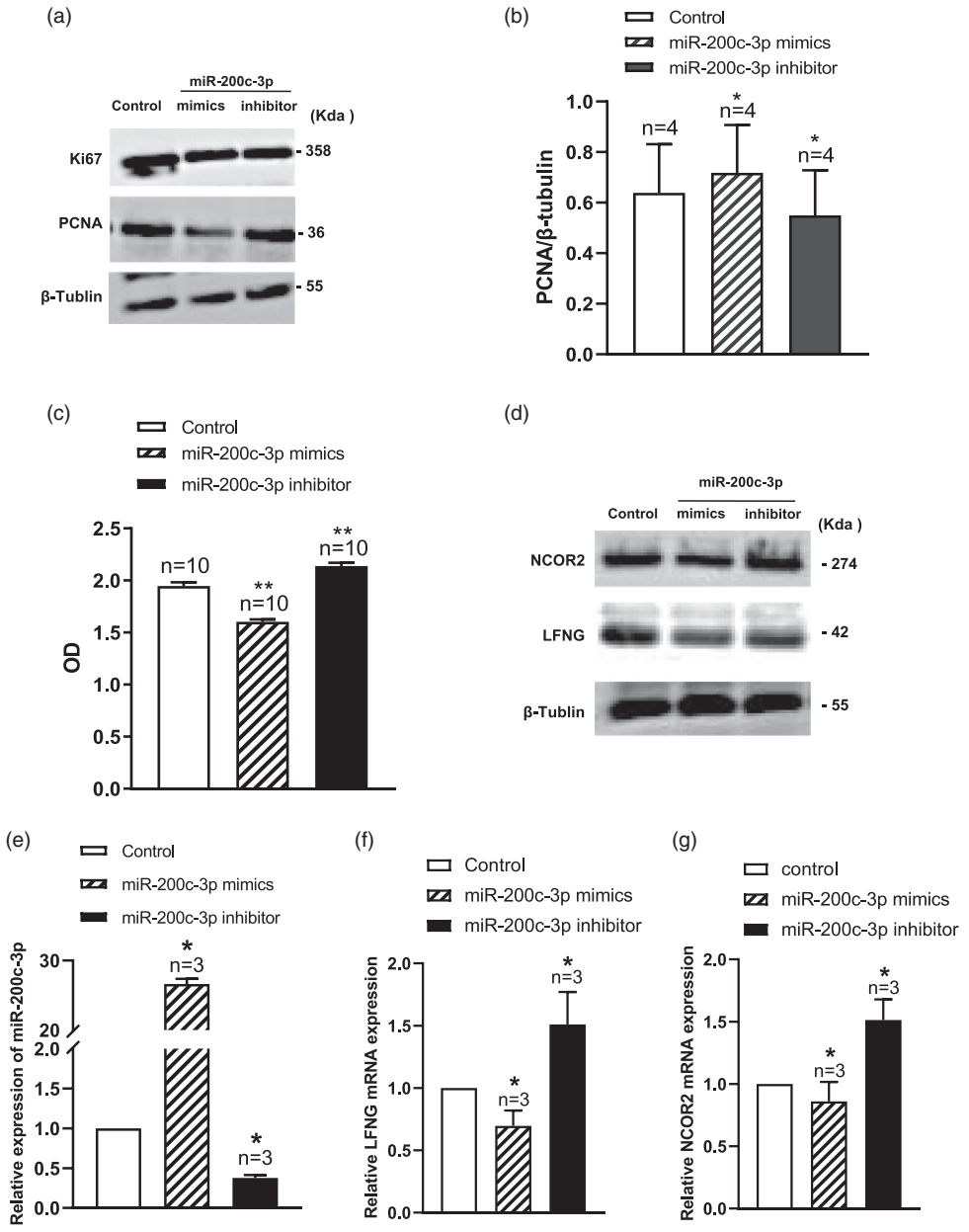

Figure 4. MiR-200c-3p affects HDMEC proliferation and the expression of NCOR2 and LFNG. (a, b) Ki67 and PCNA expression levels following the transfection of a miR-200c-3p inhibitor or mimics in human dermal microvascular endothelial cells (HDMECs). (c) HDMEC proliferation following transfection of a miR-200c-3p inhibitor or mimics. (d) NCOR2 and LFNG expression in HDMEC transfected with miR-200c-3p mimics or inhibitor was analyzed by Western blot. (e–g) Expression of miR-200c-3p, LFNG, and NCOR2 in HDMEC transfected with miR-200c-3p mimics or inhibitor was analyzed by qPCR. Data are presented as the means ± SD from the number of independent experiments indicated at the top of each bar. $^{*}P < 0.05$, $^{**}P < 0.01$.

contribute to the abnormal proliferation of endothelial cells. We transfected HDMECs with a miR-200c-3p mimics or inhibitor to overexpress or inhibit miR-200c-3p, respectively, and then detected the proliferation of HDMECs by Western blotting and CCK-8 analyses. The Western blotting results showed that the expression levels of the proliferation cell markers PCNA and Ki67 were increased following transfection with miR-200c-3p mimics and decreased following that with miR-200c-3p inhibitor (Figure 4(a) and (b)). In addition, our CCK-8 assay results revealed that transfection with miR-200c-3p inhibitor increased HDMEC proliferation, whereas transfection with a miR-200c-3p mimics deceased HDMEC proliferation (Figure 4(c)). These findings demonstrated that overexpression of miR-200c-3p inhibited the proliferation of HDMECs. The addition of the Notch signaling pathway inhibitor DAPT to HDMECs transfected with miR-200c-3p inhibitor eliminated the enhanced proliferation induced by the inhibitor in HDMECs (Figure 5(a) and (b)). Application of DAPT to cells transfected with the miR-200c-3p mimics further inhibited proliferation beyond the level caused by the mimics in HDMECs (Figure 5(a) and (b)). Thus, compared with DAPT treatment alone, the addition of DAPT treatment to transfection of HDMECs with miR-200c-3p mimics increased the inhibition of the Notch signaling pathway, and the addition of DAPT to treatment to

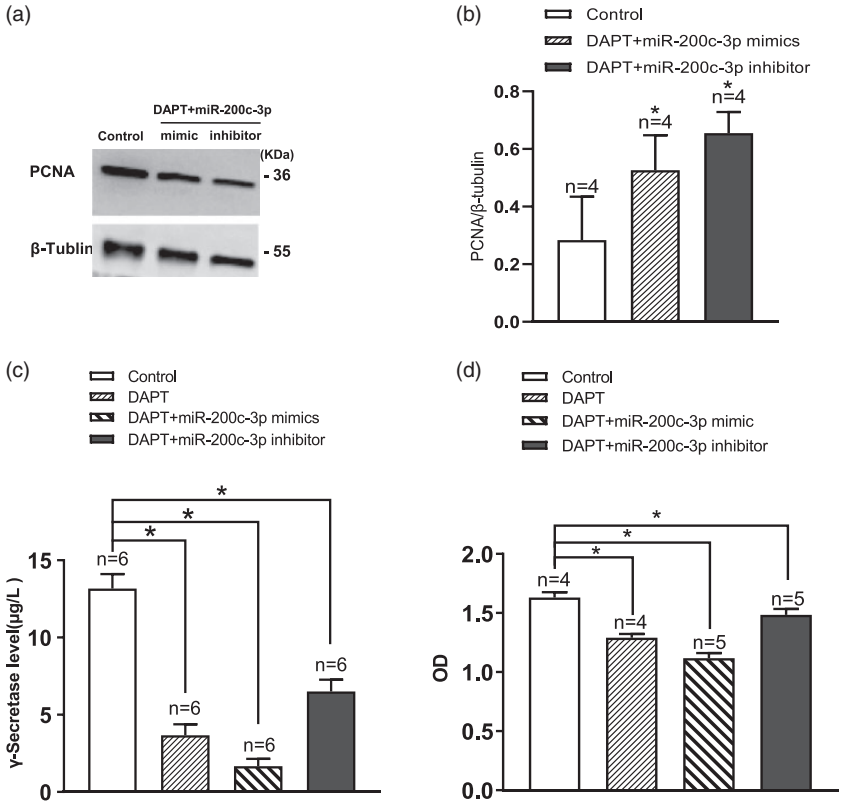

Figure 5. miR-200c-3p affects the Notch signaling pathway and HDMEC proliferation based on DAPT. (a, b) PCNA expression levels in human dermal microvascular endothelial cells (HDMECs) treated with DAPT and transfected with a miR-200c-3p inhibitor or mimics. (c) HDMEC proliferation is increased when miR-200c-3p is inhibited. (d) Cell proliferation assessed with the CCK-8 assay following treatment with DAPT in HDMECs or with DAPT applied to HDMECs transfected with either a miR-200c-3p inhibitor or mimics. Data are presented as the means ± SD from the number of independent experiments indicated above the bars; $^*P < 0.05$.

transfection of HDMECs with miR-200c-3p inhibitor enhanced the activity of Notch signaling pathway (Figure 5(c)). In addition, CCK-8 assay revealed that miR-200c-3p affected HDMEC proliferation depending on the presence of DAPT (Figure 5(d)). Taken together, these findings indicated that miR-200c-3p inhibited the activation of the Notch signaling pathway in HDMECs. However, the Notch signaling pathway was not the only target of miR-200c-3p in HDMECs. DAPT is a specific inhibitor of γ-secretase, which inhibits the Notch signaling pathway by inhibiting γ-secretase. Our research shows that miR-200c-3p can inhibit the Notch signaling pathway by inhibiting γ-secretase and by regulating the expression of LFNG and NCOR2. Compared with the traditional inhibitor DAPT, miR-200c-3p had a weaker effect on the Notch signaling pathway in HDMECs, and the mechanism of action was not exactly the same as that of DAPT.

Discussion

The present study analyzed data sets derived from GEO. We found that the expression level of miR-200c-3p was significantly and markedly lower in hemangioma tissue than in healthy tissue, and we identified 20 genes that were not only differentially expressed between infantile hemangioma and adjacent healthy tissue but also target genes of miR-200c-3p. We studied two genes involved in the Notch signaling pathway and found that miR-200c-3p inhibits the proliferation of cells in hemangioma by targeting the Notch signaling pathway. We used DAPT to treat HDMECs to verify the role of the Notch signaling pathway in hemangioma. Furthermore, we treated cells that had been transfected with miR-200c-3p mimics or inhibitor with DAPT. We found that miR-200c-3p had a weak effect relative to that of DAPT on the Notch signaling pathway in HDMECs and that the mechanism of action of miR-200c-3p was not exactly the same as that of DAPT.

The pathogenesis of infantile hemangiomas is complex and diverse. Multifactor interactions lead to the development of hemangioma, and the pathogenesis needs to be further explored.[10,11,14] MicroRNAs play important roles in the occurrence and development of many diseases, including hemangioma. For example, previous research has shown that miR−424 suppresses the proliferation of hemangioma−derived endothelial cells by targeting VEGFR−2, whereas miR-195-5p promotes the proliferation of hemangioma vascular endothelial cells.[26,27] Through bioinformatics analysis, the present study revealed that the expression of microRNA was significantly downregulated in hemangioma compared with healthy tissue.

Owing to the low number of hemangioma patients currently treated by surgery, the prognostic significance of miR-200c-3p has not yet been evaluated. Similarly, we have not obtained enough hemangioma tissue for primary hemangioma cell culture. Therefore, we used HDMEC to reveal the relationship between the abnormal proliferation of vascular endothelial cells and the occurrence and

development of hemangioma by revealing the mechanism of vascular endothelial cell proliferation. Our functional studies showed that the expression of miR-200c-3p significantly reduced HDMEC proliferation. In contrast, downregulation of miR-200c-3p promoted HDMEC proliferation. VEGF is a known pro-angiogenic factor. The expression of miR-200c-3p inhibited VEGF-induced HDMEC proliferation. Thus, miR-200c-3p plays a suppressive role in the growth of hemangiomas. Previous work has shown that miR-200c-3p is a member of the miR-200 family and is located on chromosome 12. It has been extensively studied for its roles in the development, proliferation, therapy resistance, and metastasis of cancers.[19–22] In addition, miR-200c-3p can coordinate Notch signaling and VEGF signaling to influence tumor cell proliferation.[28] The Notch signaling system plays an important role in angiogenesis, both in general and in hemangioma. VEGF is an upstream regulator of Notch signaling. When VEGFR is activated, it downregulates the Notch signaling pathway receptor Notch-1 and delta-like ligand 4 expression in vascular endothelial cells and promotes vascular development and proliferation.[29]

To better understand the mechanisms by which miR-200c-3p inhibits HDMEC proliferation, we investigated the target genes of miR-200c-3p that are involved in this process. We found that overexpression of miR-200c-3p reduced endogenous expression of two genes involved in the Notch signaling pathway (*LFNG* and *NCOR2*) in HDMECs. Inhibiting the Notch signaling pathway produced an effect on HDMEC proliferation similar to that of miR-200c-3p overexpression. These results supported the inhibition of the Notch signaling pathway as an important mechanism of miR-200c-3p-mediated inhibition of HDMEC proliferation. This study only conducted functional analyses at the level of normal vascular endothelial cells. Additional studies on hemangioma cells and *in vivo* animal experiments are needed to further explore the relationship between miR-200c-3p and the occurrence and development of hemangioma.

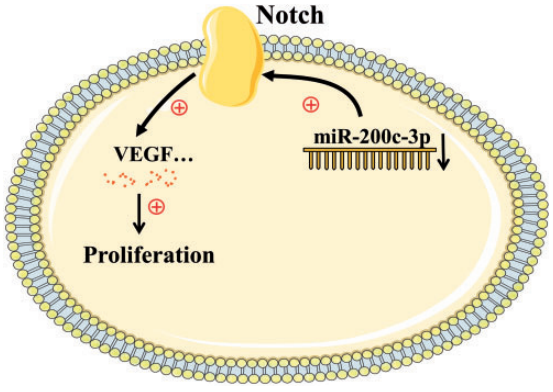

Figure 6. Schematic diagram showing the proposed signaling complex transduction pathway. miR-200c-3p inhibits the expression of the Notch signaling pathway in cells, and the low expression of miR-200c-3p in HDMEC reduces the inhibitory effect on the Notch signaling pathway, further affecting the proliferation of HDMECs in infantile hemangioma. (A color version of this figure is available in the online journal.)

Conclusions

In summary, as shown in Figure 6, the present study showed that miR-200c-3p expression is downregulated in hemangiomas. The downregulation of miR-200c-3p increased HDMEC proliferation through the Notch signaling pathway. Thus, our study demonstrates that the Notch signaling pathway is a target of miR-200c-3p. Understanding the role of miR-200c-3p in HDMEC proliferation will provide a basis for developing miR-200c-3p as a potential therapeutic target for infantile hemangioma.

AUTHORS' CONTRIBUTIONS

XH, SB, JD, and SL developed the methodological aspects of the analysis and data interpretation. SB, XY, and ZN analyzed the data, prepared the figures, and drafted the manuscript. XH, PT, and BS drafted the manuscript. All authors revised the manuscript, approved the final version, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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DECLARATION OF CONFLICTING INTERESTS

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ORCID iDs

Xianyu Hu https://orcid.org/0000-0002-0734-1989
Bing Shen https://orcid.org/0000-0001-7263-4748

SUPPLEMENTAL MATERIAL

Supplemental material for this article is available online.

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