miRNA-29a inhibits the proliferation of HUVECs by regulating the ITGB1/β-catenin/c-Myc pathway

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Abstract. Infantile hemangioma (IH) is a type of benign vascular tumor observed in younger patients. Previously, 216 differentially expressed microRNAs (miRs/miRNAs) associated with IH have been identified. In addition, common hub genes and miRNAs related to proteoglycan signaling pathways in angiogenesis and cancer have been identified, including c-Myc, integrin β1 (ITGB1), Bcl2 and miR-29a. Therefore, the present study aimed to explore the pathogenesis of IH from the perspective of previously identified miRNA gene network and protein-protein interactions. Gene and protein levels in human umbilical vein endothelial cells (HUVECs) were analyzed using reverse transcription-quantitative PCR and western blot (WB) analysis. Cell viability was assessed using a Cell Counting Kit-8 assay, and the potential association between miR-29a with ITGB1 was validated using a dual-luciferase reporter assay. The inhibition of ITGB1 suppressed the β-catenin/c-Myc pathway in HUVECs. In addition, transfection with small interfering RNAs (siRNAs) targeting ITGB1 decreased the viability of HUVECs. Furthermore, siRNAs targeting mucin 1 and β-N-acetylglucosaminidase significantly inhibited the c-Myc pathway in HUVECs. The results of WB and dual-luciferase reporter assays demonstrated that miR-29a regulated the β-catenin/c-Myc pathway and the viability of HUVECs in HUVECs by directly binding to ITGB1. Therefore, miR-29a may serve as a potential therapeutic target for IH.

Introduction

Infantile hemangioma (IH) is one of the most prevalent benign vascular tumors in infants and young children (1). It is comprised of benign proliferating vascular endothelial cells, and the prevalence rate of IH is 5-10% globally (2). Preterm birth, low birth weight, multiple pregnancies, progesterone use during pregnancy and a family history of the disease are potential risk factors (1-3). IH is characterized by the onset of bright red papules or nodules 2-4 weeks after birth. This rapid growth period lasts for ~5 months, reaching a plateau until ~1 year of age and finally entering a slow regression period; the vast majority of IHs eventually break down into fibrous fat residues between 5 and 10 years of age (4). Although IH is benign and eventually fades, rapid tumor growth during proliferation can occasionally lead to disfigurement and life-threatening complications, including ulcers, bleeding, permanent visual impairment, airway obstruction leading to respiratory distress and congestive heart failure (5,6). Therefore, early intervention in the rapid proliferative stage of IH is key to minimizing or preventing the risk of developing potential complications. The current clinical treatments for IH include the use of β-blockers, corticosteroids, interferon, vincristine, angiotensin-converting enzyme inhibitors, laser therapy and surgical treatment (7,8). However, since the pathogenesis of IH remains to be fully elucidated, these treatments are only partially effective or can result in severe adverse reactions (7).

MicroRNAs (miRNAs/miRs) are endogenous, small, non-coding RNA molecules that can regulate gene expression by directly binding with target mRNAs (9,10). In our previous study, RNA sequencing screening was performed for differentially expressed miRNAs related to IH, resulting in the identification of 97 upregulated and 119 downregulated miRNAs (11). In addition, the enrichment and pathways of potential target genes were analyzed, and it was found that the proteoglycans in cancer signaling pathway exhibited a notable difference. Common hub genes and miRNAs identified as being related to proteoglycan signaling pathways in angiogenesis and cancer, including c-Myc, integrin β1 (ITGB1), Bcl2 and miR-29a, were screened through an miRNA gene network and protein-protein interaction analysis. The results of the RNA sequencing screening suggested that c-Myc, ITGB1, Bcl2 and miR-29a may have served important roles during the pathogenesis of IH (11).

Integrins are members of the cell surface adhesion protein receptor family, consisting of 18 specific α subunits and eight unique β subunits, which form 24 heterodimer structures in a non-covalent binding manner (12). Each heterodimer can bind

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to specific sequences of extracellular matrix, cell surface or soluble protein ligands. Integrins are involved in a number of biological processes, such as cell polarity, cell surface adhesion, proliferation, apoptosis, tumor formation and metastasis, and are key proteins in the connection between cells and the surrounding environment (13). ITGB1 has been shown to serve a carcinogenic role in a variety of types of cancer. The Akt/Wnt/β-catenin pathway is a key downstream regulator of ITGB1 carcinogenesis in a variety of tumors (14,15), while c-Myc is a downstream target of the Wnt/β-catenin pathway (16). c-Myc has also been shown to be carcinogenic in various types of cancer (17,18). A previous study revealed the association between the ITGB1/Wnt/β-catenin/c-Myc axis and ITGB1-divergent transcript (ITGB1-DT), a long non-coding RNA located on chromosome 10p11.22, for which the direction of transcription is opposite to that of the coding gene ITGB1; ITGB1-DT interacts with histone-lysine N-methyltransferase EZH2 (EZH2), inhibits the binding of EZH2 to the ITGB1 promoter and reduces the trimethylation level of lysine 27 on histone H3 in the ITGB1 promoter region, resulting in the activation of ITGB1 expression (19). At the same time, the upregulation of ITGB1 activates the Wnt/β-catenin/c-Myc pathway, which in turn directly transcriptionally activates ITGB1-DT expression (6,19). It has therefore been suggested that ITGB1 can transcriptionally upregulate c-Myc mRNA expression through the Wnt/β-catenin pathway. In addition, mucin 1 (MUC1), a transmembrane glycoprotein involved in cell regeneration, differentiation, adhesion, integration, signaling and apoptosis, has been reported to activate and regulate the Wnt/β-catenin pathway (20).

Similar to phosphorylation, O-linked β-N-acetylglucosaminylation (O-GlcNAcylation) is a broad and dynamic protein modification regulated by β-N-acetylglucosaminidase (OGA) and O-linked N-acetylglucosamine transferase 110 kDa subunit (OGT) and is involved in gene transcription, signal transduction, proteolytic hydrolysis and other cell activities (21). In addition, a variety of tumor tissues exhibit high O-GlcNAcylation levels, and O-GlcNAcylation has been reported to effect cancer cell proliferation, angiogenesis and metastasis in various cancer types (22,23). It has also been reported that O-GlcNAcylation can promote the stability of the c-Myc protein by interfering with ubiquitination and proteasome-mediated c-Myc degradation (24,25).

It has been reported that the high expression of c-Myc in IH promotes the proliferation of hemangioma endothelial cells (11). As such, it is possible that miR-29a may affect angiogenesis of hemangioma endothelial cells by regulating the c-Myc pathway. Therefore, the present study explored the interactions between miR-29a, the c-Myc pathway and the pathogenesis of angiogenesis using small interfering RNA (siRNA) interference, reverse transcription-quantitative PCR (RT-qPCR) and western blot (WB) analysis with the aim of providing a basis for novel strategies targeting IH.

Materials and methods

Cells and transfections. Human umbilical vein endothelial cells (HUVECs) represent a commonly-used *in vitro* cell model for IH (26). HUVECs were confirmed to be mycoplasma free by using a mycoplasma analysis kit (Wuhan Servicebio Technology Co., Ltd.). As such, HUVECs were purchased from the American Type Culture Collection (cat. no. CRL-1730) and cultured in EGM™ Medium (Lonza Group, Ltd.) containing 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) with 1% penicillin-streptomycin solution (Beyotime Biotechnology) in a humidified incubator at 37˚C with 5% CO_2. HUVECs were confirmed to be mycoplasma free. Cells were used in the transfection experiments upon reaching 80% confluency (passage 6). HUVECs were then transfected with 20 nM of the relevant siRNA. The siRNA sequences used were as follows: ITGB1 siRNA1, sense 5'-GCC UUGCAUUACUGCUGAUAU-3', antisense 5'-AUAUCAGCA GUAAUGCAAGGC-3; ITGB1 siRNA2, sense 5'-GCCCUC CAGAUGACAUAGAAA-3', antisense 5'-UUUCUAUGU CAUCUGGAGGGC-3'; MUC1 siRNA1, sense 5'-GACACA GUUCAAUCAGUAUAA-3', antisense 5'-UUAUACUGA UUGAACUGUGUC-3'; MUC1 siRNA2, sense 5'-CCGGGA UACCUACCAUCCUAU-3', antisense 5'-AUAGGAUGGUAG GUAUCCCGG-3'; OGT siRNA1, sense 5'-UUUAGCACU CUGGCAAUUAAA-3', antisense 5'-UUUAAUUGCCAG AGUGCUAAA-3; OGT siRNA2, sense 5'-GCUGAGCAG UAUUCCGAGAAA-3', antisense 5'-UUUCUCGGAAUA CUGCUCAGC-3'; and siRNA negative control, sense 5'-AUC UUUGAUAUCGCGUCUACG-3, anti-sense 5'-CGUAGA CGCGAUAUCAAAGAU-3'. Cells were transfected for 6 h at 37˚C using Lipofectamine® 2000 reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and were then cultured for a further 48 h before subsequent experiments were performed. All siRNAs and the negative control were provided by Guangzhou RiboBio Co., Ltd.

In addition, HUVECs were also transfected with 20 μg/ml miR-29a mimics, inhibitors or negative controls as appropriate. The sequences were manufactured by Guangzhou RiboBio Co., Ltd. and were as follows: miR-29a mimic, sense 5'-UAGCACCAUCUGAAAUCGGUUA-3', antisense 5'-ACCGAUUUCAGAUGGUGCUAUU-3'; miR-29a mimic negative control, sense 5'-UUCUCCGAACGUGUCACG UUU-3', antisense 5'-AAACGUGACACGUUCGGAGAA-3'; miR-29a inhibitor 5'-UAACCGAUUUCAGAUGGUGCUA-3'; and miR-29a inhibitor negative control, 5'-CAGUACUUU UGUGUAGUACAA-3'. HUVECs were transfected with the aforementioned miR-29a mimic and inhibitor sequences for 6 h using Lipofectamine 2000® and then cultured for a further 48 h before subsequent experimentation.

Cell viability analysis. A Cell Counting Kit-8 (CCK-8; Nanjing KeyGen Biotech Co., Ltd.) assay was used to assess cell viability. Cells were seeded into a 96-well plate and subjected to the aforementioned transfection protocol; subsequently, transfected HUVECs were incubated with CCK-8 reagent for 2 h at 37˚C. The absorbance of samples was measured at 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.).

RT-qPCR. RNA was extracted from the HUVECs using Trizol (Ambion; Thermo Fisher Scientific, Inc.). Primarily, RNA was reverse transcribed into cDNA using HiScript Reverse Transcriptase (Vazyme Biotech Co., Ltd.) according to the manufacturer's protocol. The mRNA expression level was then determined using SYBR qPCR Master Mix (Vazyme Biotech Co., Ltd.) alongside specific primers (Table I) on

Table I. Primer sequences for reverse transcription-quantitative PCR.

Gene	Forward, 5'-3'	Reverse, 5'-3'
ITGB1	TCCAACCTGATCCTGTGTCC	CAATTCCAGCAACCACACCA
ITGB1-DT	CAAAACCTGAAGCCCCAAAGA	CACTGCACCGTCTTCCTAATG
c-Myc	AACACACAACGTCTTGGAGC	GCACAAGAGTTCCGTAGCTG
EZH2	CGGCTTCCCAATAACAGTAGC	ACTCCACTCCACATTCTCAGG
MUC1	GTGATGTGCCATTTCCTTTCTCT	TCGCTCATAGGATGGTAGGTATC
OGT	AGGTGTTCTGTTATGCCCTGA	AGCGCCCTTAGTATAGCCATT
OGA	CAGCCTGATGAAGAACCCATG	TGTTCATCAGTTTGCATGGGG
GAPDH	TCAAGAAGGTGGTGAAGCAGG	TCAAAGGTGGAGGAGTGGGT
U6	CGCTTCGGCAGCACATATAC	AAATATGGAACGCTTCACGA
hsa-miR-29a-3p[a]	TGCGCTAGCACCATCTGAAATCGG	CCAGTGCAGGGTCCGAGGTATT

[a]Stem-loop primer of hsa-miR-29a-3p, 5'-GTCGTATCCAGTGCAGGGTCCGAGGTATTCGCACTGGATACGACTAACCGAT-3'. ITGB1, integrin β1; ITGB1-DT, integrin subunit β1 divergent transcript; EZH2, histone-lysine N-methyltransferase EZH2; MUC1, mucin 1; OGT, O-linked N-acetylglucosamine transferase 110 kDa subunit; OGA, β-N-acetylglucosaminidase; hsa-miR, human microRNA.

an eQ9600 PCR System (Eastwin Life Sciences; Beijing Dongsheng Innovation Biotechnology Co., Ltd.). The thermocycling conditions were as follows: Pre-incubation at 95˚C for 30 sec; amplification at 95˚C for 10 sec and 60˚C for 30 sec, for 40 cycles. The relative mRNA levels were quantified using the $2^{-\Delta\Delta Cq}$ method with GAPDH as the housekeeping gene (27).

WB. The total protein content of HUVECs was extracted using RIPA lysate (cat. no. P0013C; Beyotime Biotechnology) and electrophoresed on 10% SDS-PAGE (10 μg/lane protein), before proteins were transferred onto PVDF membranes (MilliporeSigma; Merck KGaA). The protein concentration was measured with the BCA method (cat. no. P0010; Beyotime Biotechnology). The membranes were blocked with 5% skimmed milk for 1 h at room-temperature and incubated with the following primary antibodies overnight at 4˚C: ITGB1 (cat. no. 12594-1-AP; Proteintech Group, Inc.), T-cell factor 1 (TCF1; cat. no. 14464-1-AP; Proteintech Group, Inc.), EZH2 (cat. no. ab307646; Abcam), c-Myc (cat. no. 67447-1-Ig; Proteintech Group, Inc.), phosphorylated (p-)GSK3β (cat. no. AF2016; Affinity Biosciences), GSK3β (cat. no. AF5016; Affinity Biosciences), p-β-catenin (cat. no. DF2989; Affinity Biosciences), β-catenin (cat. no. 17565-1-AP; Proteintech Group, Inc.), β-actin (cat. no. 66009-1-Ig; Proteintech Group, Inc.), OGT (cat. no. ab177941; Abcam) and OGA (cat. no. DF8436; Affinity Biosciences). All primary antibodies were used at a dilution of 1:1,000. Subsequently, the membranes were incubated with HRP-labeled goat anti-rabbit secondary antibody (1:5,000; cat. no. ab6721; Abcam) or goat anti-mouse secondary antibody (1:5,000; cat. no. ab6789; Abcam) for 1 h at room temperature. Finally, the membrane signals were detected using an ECL substrate kit (Thermo Fisher Scientific, Inc.) and quantified using ImageJ software 1.8.0 (National Institutes of Health).

Dual-luciferase reporter assay. The bioinformatics tool TargetScan (https://www.targetscan.org/vert_80/) was used to predict the potential association between miR-29a and ITGB1. The ITBG1 3'-untranslated region sequences containing the wild-type or mutant miR-29a binding sequence were inserted into the pGL6 luciferase reporter (Beyotime Biotechnology). HUVECs were then co-transfected with the luciferase reporter and the miR-29a mimic or mimic negative control using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.). After 48 h transfection at 37˚C, the cells were harvested for luciferase activity detection according to the instruction of the Dual-Luciferase® Reporter Assay kit used (Promega Corporation). *Renilla* luciferase activity was used to normalize reporter activity.

Statistical analysis. All experiments were performed three times (n=3). The data are presented as mean ± SD and analyzed using GraphPad Prism software (version 7; Dotmatics). One-way ANOVA with Tukey's post hoc test was used to analyze the results when comparing >2 groups in the present study, and a value of $P<0.05$ was considered to indicate a statistically significant difference.

Results

Inhibition of ITGB1 downregulates the β-catenin/c-Myc pathway. The present study first explored the effects of ITGB1 on the c-Myc pathway in HUVECs using ITGB1 siRNAs. As indicated by the results of RT-qPCR, both ITGB1 siRNA1 and siRNA2 significantly inhibited the gene expression of ITGB1 in HUVECs compared with control HUVECs and the control siRNA (Fig. 1A). In addition, both ITGB1 siRNA1 and siRNA2 significantly inhibited the gene expression levels of ITGB1-DT, EZH2 and c-Myc in HUVECs (Fig. 1B-D). Consistently, the results of WB analysis demonstrated that both ITGB1 siRNA1 and siRNA2 significantly decreased ITGB1, EZH2, c-Myc, p-GSK3β and p-β-catenin protein expression levels in HUVECs compared with the controls (Fig. 1E and F). The CCK-8 assay also revealed that a significant decrease in cell viability was observed in HUVECs transfected with ITGB1 siRNA sequences compared with both controls (Fig. 1G). On the whole, these data suggest that inhibition of ITGB1 is able to suppress the β-catenin/c-Myc pathway in HUVECs.

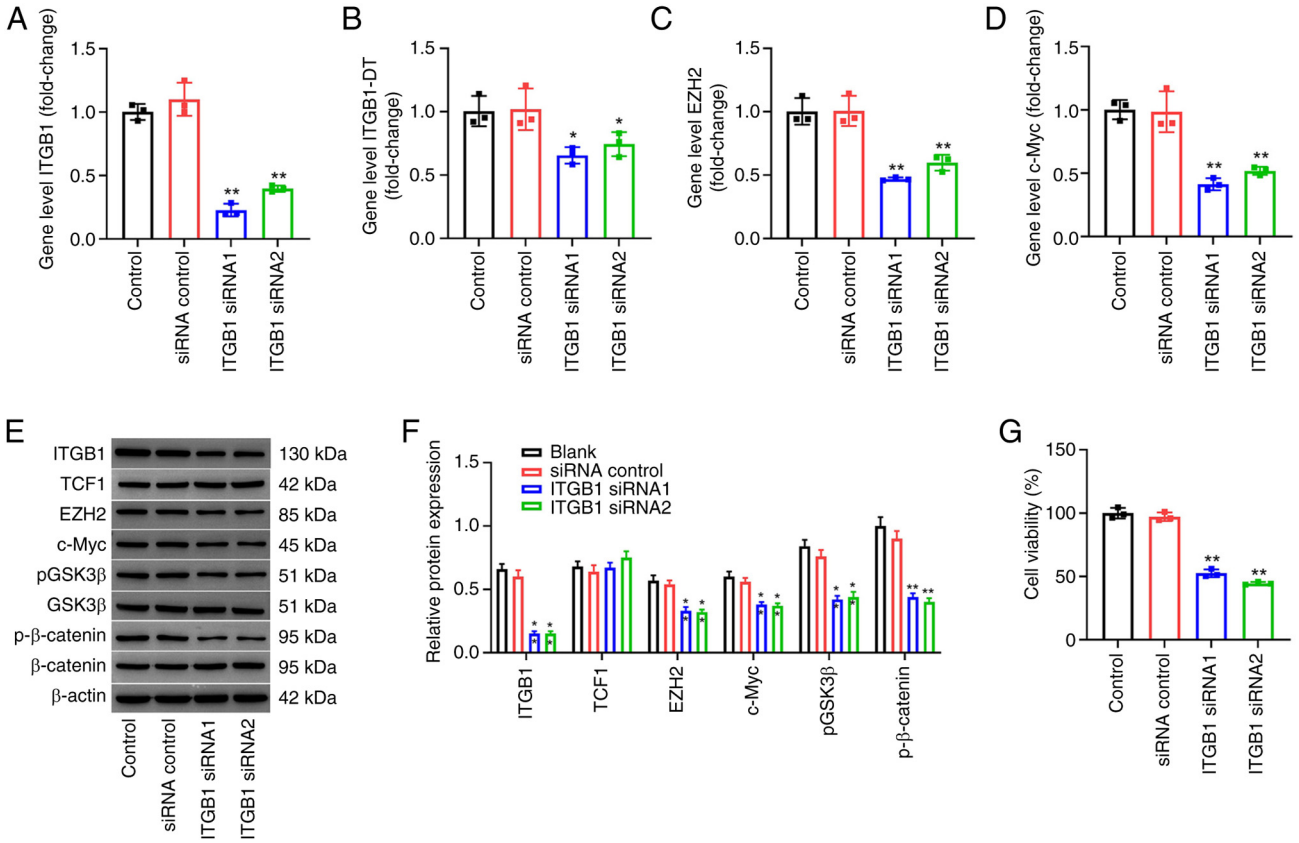

Figure 1. Inhibition of ITGB1 downregulates the β-catenin/c-Myc pathway in HUVECs. (A) ITGB1 gene expression after HUVECs were transfected with 20 nM ITGB1 siRNA1, siRNA2 or siRNA negative control for 6 h, and cultured for another 48 h. The gene expressions of (B) ITGB1-DT, (C) EZH2 and (D) c-Myc were assessed using reverse transcription-quantitative PCR following transfection with ITGB1 siRNA. (E) The protein expression levels of ITGB1, TCF1, EZH2, c-Myc, p-GSK3β, GSK3β, p-β-catenin and β-catenin were evaluated using western blotting following transfection with ITGB1 siRNA. (F) Semi-quantification of western blotting results. p-GSK3β and p-β-catenin were normalized to the levels of total GSK3β or β-catenin, respectively. (G) Cell viability was analyzed using a Cell Counting Kit-8 assay. n=3; *P<0.05 and **P<0.01 compared with siRNA control group. HUVECs, human umbilical vein endothelial cells; ITGB1, integrin β1; ITGB1-DT, integrin subunit β1 divergent transcript; EZH2, histone-lysine N-methyltransferase EZH2; siRNA, small interfering RNA; TCF1, T-cell factor 1; p-, phosphorylated.

Inhibition of MUC1 suppresses the c-Myc pathway in HUVECs. The function of MUC1 was subsequently explored using RT-qPCR and CCK-8 assays. The results indicated that the inhibition of MUC1 using MUC1-targeting siRNA significantly downregulated c-Myc gene expression in HUVECs (Fig. 2A and B). In addition, significantly decreased cell viability was observed in the HUVECs transfected with both MUC1 siRNA sequences compared with the controls (Fig. 2C). These results suggest that the inhibition of MUC1 inhibits c-Myc expression and the viability of HUVECs.

Inhibition of OGT downregulates the c-Myc pathway in HUVECs. The present study then investigated the role of OGT in HUVECs using OGT-targeting siRNAs. As shown by the results of RT-qPCR, both OGT siRNA sequences significantly inhibited the gene expression of OGT in HUVECs (Fig. 3A). In addition, both OGT siRNA sequences significantly decreased the gene expression levels of OGA and c-Myc in HUVECs compared with controls (Fig. 3B and C). Consistent with the data obtained using RT-qPCR, both OGT siRNA sequences significantly decreased OGT, OGA and c-Myc protein expression levels in HUVECs compared with control expression levels (Fig. 3D and E). The CCK-8 assay suggested that, compared with control groups, transfection with OGT siRNA significantly decreased the viability of HUVECs (Fig. 3F). Summarily, these data suggest that OGT-mediated O-GlcNAcylation is able to stabilize the c-Myc protein and contribute to the upregulation of c-Myc in HUVECs.

miR-29a inhibits ITGB1 gene expression. In our previous study, c-Myc, ITGB1, Bcl2 and miR-29a were identified to be associated with proteoglycan signaling in angiogenesis (11). In the present study, miR-29a expression in HUVECs was significantly upregulated by miR-29a mimics and significantly downregulated by application of an miR-29a inhibitor compared with the control groups (Fig. 4A). Additionally, transfection with the miR-29a mimic significantly inhibited ITGB1, ITGB1-DT, MUC1 and c-Myc gene expression levels compared with controls; furthermore, the miR-29a inhibitor exerted opposing effects to the miR-29a mimic on these proteins by significantly upregulating their expression compared with the controls (Fig. 4B-E). The transfection efficiency of the miR-29a mimic and miR-29a inhibitor was evaluated compared with specific negative controls for the mimic and inhibitor respectively in Fig. 4A, but all other experiments were performed with a negative control for the mimic alone. These results provide evidence that miR-29a was involved in the angiogenesis of HUVECs.

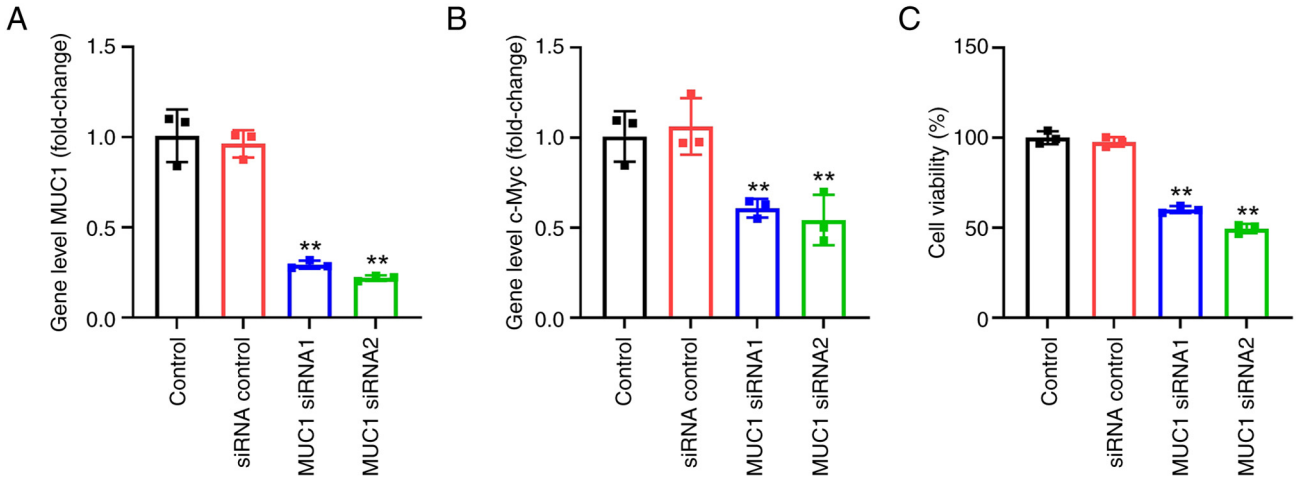

Figure 2. Inhibition of MUC1 downregulates the c-Myc pathway in HUVECs. HUVECs were transfected with 20 nM MUC1 siRNA1, siRNA2 or siRNA negative control for 6 h and cultured for another 48 h. The gene expressions of (A) MUC1 and (B) c-Myc were assessed using reverse transcription-quantitative PCR. (C) Cell viability was analyzed using a Cell Counting Kit-8 assay. n=3; **P<0.01 compared with siRNA control group. HUVECs, human umbilical vein endothelial cells; siRNA, small interfering RNA; MUC1, mucin 1.

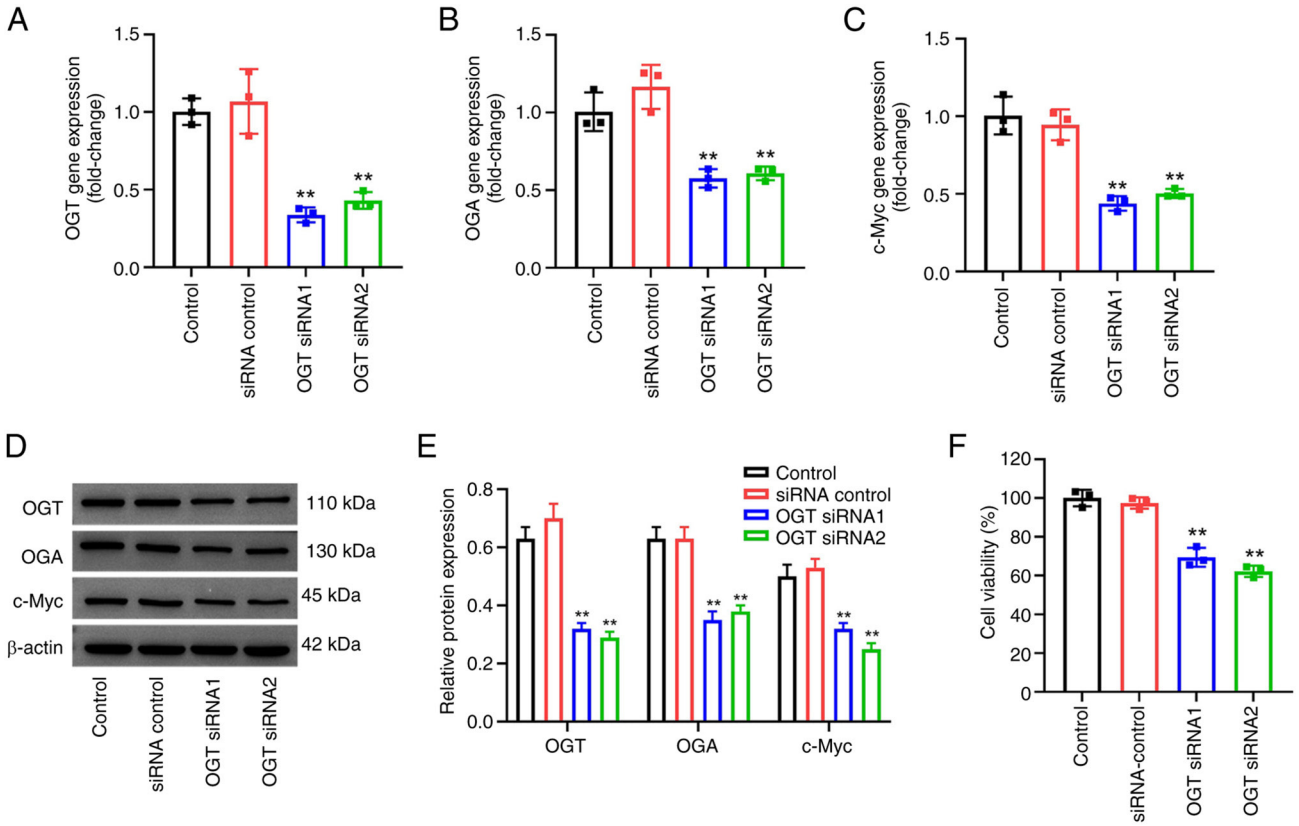

Figure 3. Inhibition of OGT downregulates the c-Myc pathway in HUVECs. HUVECs were transfected with 20 nM OGT siRNA1, siRNA2 or siRNA negative control for 6 h and cultured for another 48 h. The gene expressions of (A) OGT, (B) OGA and (C) c-Myc were detected using reverse transcription-quantitative PCR. (D) OGT, OGA and c-Myc protein expression levels were assessed using western blotting and (E) semi-quantified. (F) Cell viability was analyzed via Cell Counting Kit-8 assay. n=3; **P<0.01 compared with siRNA control group. HUVECs, human umbilical vein endothelial cells; OGT, O-linked N-acetylglucosamine transferase 110 kDa subunit; OGA, β-N-acetylglucosaminidase; siRNA, small interfering RNA.

miR-29a suppresses the β-catenin/c-Myc pathway in HUVECs by targeting ITGB1. Finally, the effects of miR-29a on the β-catenin/c-Myc pathway were explored using WB and dual-luciferase reporter analysis. The results of the WB revealed that transfection with the miR-29a mimic significantly reduced the protein expression levels of OGT, OGA, ITGB1, TCF1, c-Myc, p-GSK3β and p-β-catenin in HUVECs (Fig. 5A and B); however, the miR-29a inhibitor exerted opposing effects to the miR-29a mimic on these RNA levels, significantly upregulating the expression of OGT, OGA, ITGB1, TCF1, c-Myc and p-β-catenin compared with the control groups. Additionally, the miR-29a mimic significantly

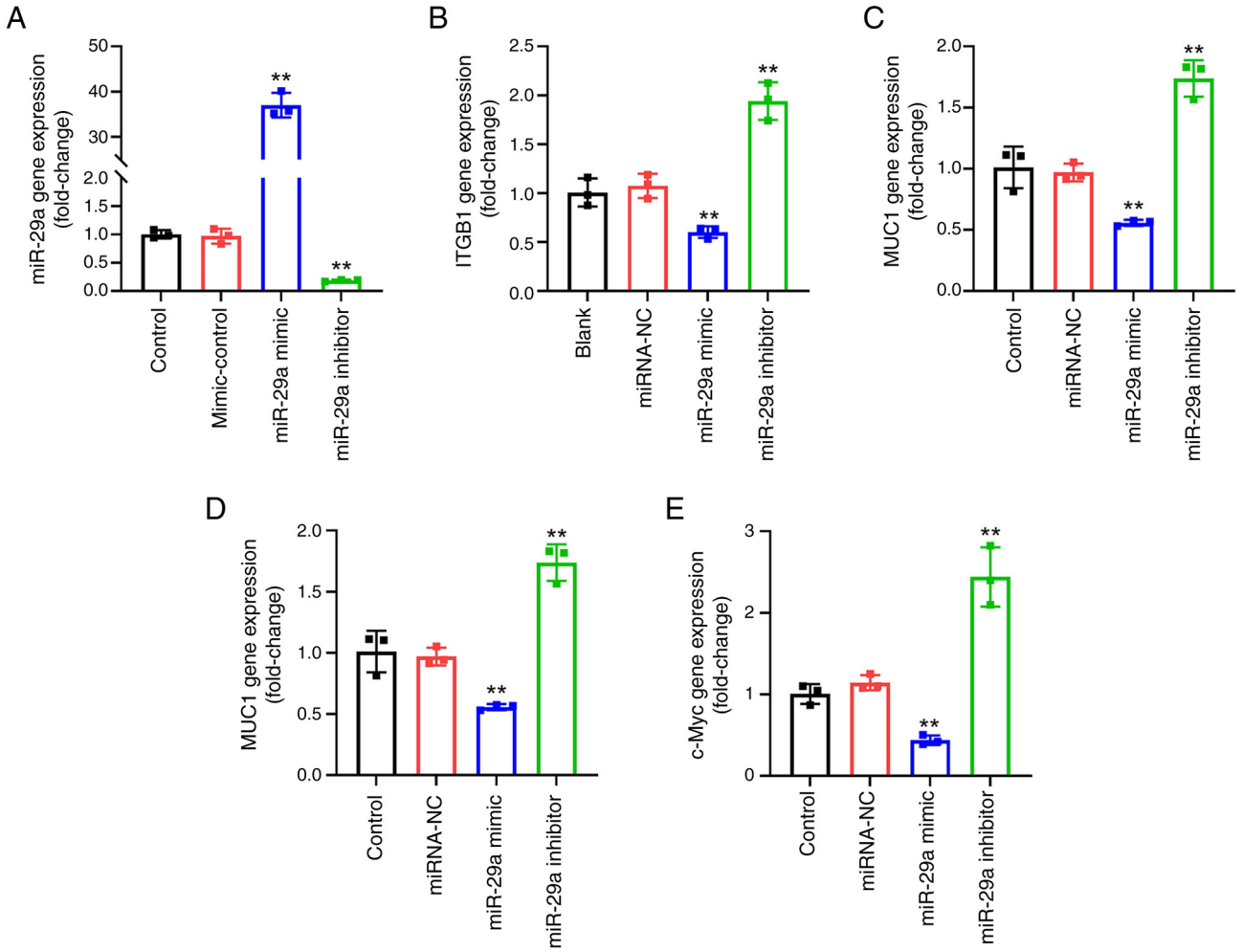

Figure 4. miR-29a inhibits ITGB1 gene expression. HUVECs were transfected with 20 μg/ml miR-29a mimic, mimic NC, miR-29a inhibitor or inhibitor control for 6 h and cultured for another 48 h. The gene expression levels of (A) miR-29a (**P<0.01 compared with miRNA-NC or inhibitor-ctrl group respectively), (B) ITGB1, (C) ITGB1-DT, (D) MUC1 and (E) c-Myc were assessed using reverse transcription-quantitative PCR. n=3; **P<0.01 compared with the miRNA-NC group. miR/miRNA, microRNA; ITGB1, integrin β1; HUVECs, human umbilical vein endothelial cells; NC, negative control; miRNA-NC, miR-29a mimic negative control; ITGB1-DT, integrin subunit β1 divergent transcript; MUC1, mucin 1; inhibitor-ctrl, miR-29a inhibitor negative control.

decreased cell viability, while the miR-29a inhibitor significantly increased the viability of HUVECs compared with controls (Fig. 5C). The data obtained from the dual-luciferase reporter assay supported the direct binding of miR-29a with wild-type ITGB1, as observed by the significant decrease in luciferase activity in the miR-29a mimic + ITGB1 wild-type group compared with controls (Fig. 5D and E). In summary, these data suggest that miR-29a suppresses the β-catenin/c-Myc pathway in HUVECs by targeting ITGB1.

Discussion

miR-29a-3p serves an important role in cell proliferation and inflammation (28). A study by Qi *et al* (29) showed that miR-29a-3p inhibited the development of osteosarcoma through modulating insulin-like growth factor 1. Additionally, a study by Cao *et al* (30) reported that miR-29a exerted apoptotic effects on HUVECs by inhibiting the PI3K/Akt/Bcl2 axis. Consistent with these reports, the present study demonstrated that ITGB1 was negatively regulated by miR-29a at the post-transcriptional level in HUVECs. In addition, miR-29a mimics significantly reduced cell viability and downregulation of the c-Myc and p-β-catenin pathways in HUVECs.

ITGB1 is the largest subgroup in the integrin family and the most important integrin protein expressed by tumor cells, which is associated with various biological behaviors exhibited by tumor cells (19,31). The upregulation of ITGB1 activates the Wnt/β-catenin pathway, which in turn directly transcriptionally activates ITGB1-DT expression, forming a positive feedback loop of ITGB1/Wnt/β-catenin/c-Myc (19). A study by Wang *et al* (32) found that Ras-related protein Rab-25 promoted erlotinib resistance by activating the ITGB1/β-catenin pathway in lung cancer. In addition, another study by Zou *et al* (33) reported that platelets facilitated metastasis of the breast cancer cell line MCF-7 by activating the integrin α2β1/Wnt/β-catenin pathway. Similarly, the present study provided evidence that miR-29a mimics inhibited the mRNA level of ITGB1 via direct binding. Subsequently, ITGB1 was unable to transcriptionally downregulate c-Myc mRNA through the Wnt/β-catenin pathway in HUVECs (Fig. 5F).

Mucin is the main component of mucus secretion, which can be divided into a membrane-associated form, which interacts with the cell surface, and a secreted subgroup based

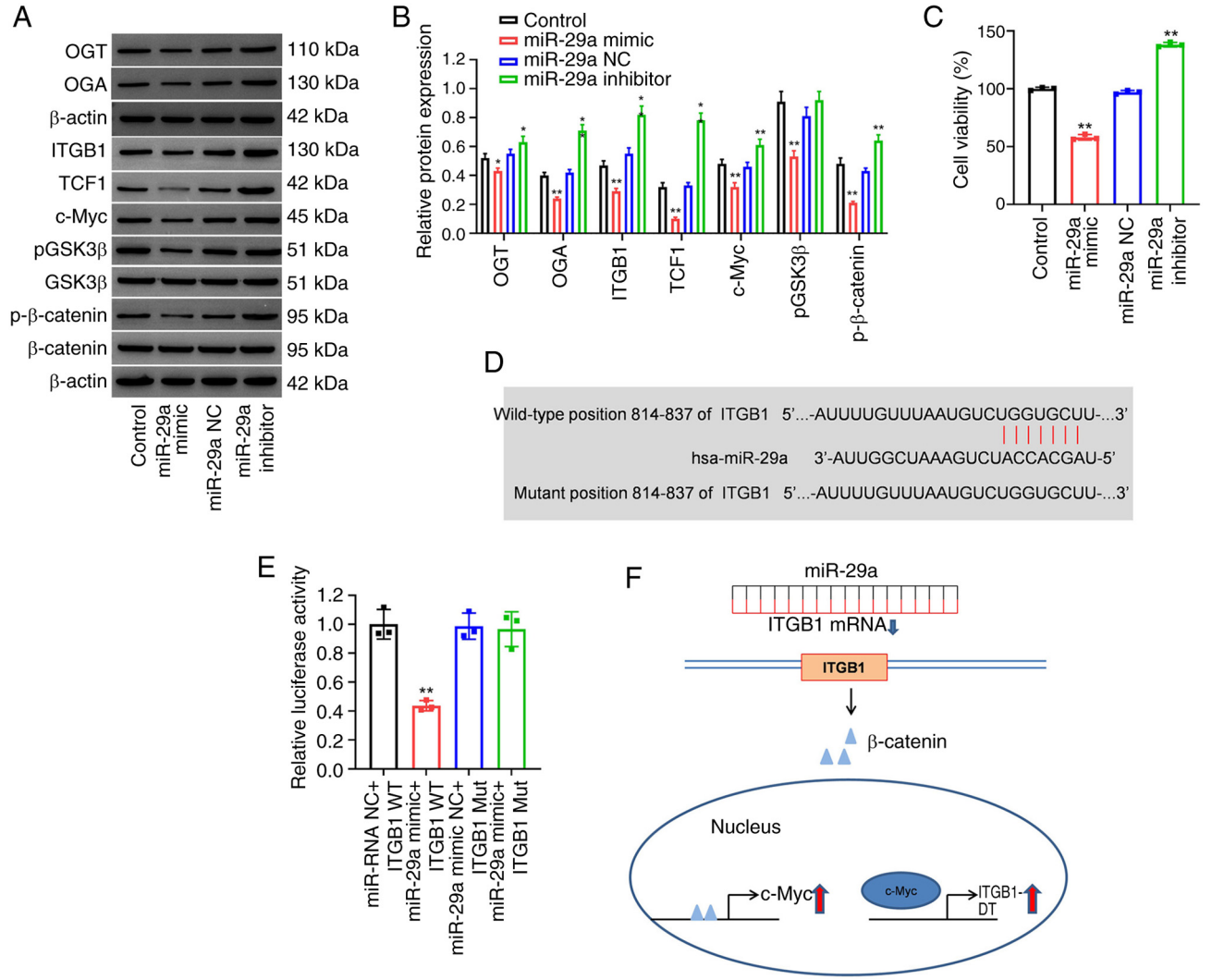

Figure 5. miR-29a downregulates the β-catenin/c-Myc pathway in HUVECs by targeting ITGB1. HUVECs were transfected with 20 μg/ml miR-29a mimics, miR-29a inhibitor or negative control for 6 h and cultured for another 48 h. (A) The protein expression levels of OGT, OGA, ITGB1, TCF1, c-Myc, p-GSK3β, GSK3β, p-β-catenin and β-catenin were assessed using western blotting and (B) semi-quantified. p-GSK3β and p-β-catenin were normalized to the levels of total GSK3β or β-catenin, respectively. (C) Cell viability was analyzed using a Cell Counting Kit-8 assay. The interaction between miR-29a and ITGB1 was validated using (D) TargetScan and (E) via dual-luciferase reporter assay (**P<0.01 compared with miR NC + ITGB1 WT group). (F) A schematic figure illustrating the interactions among miR-29a, ITGB1 and the c-Myc pathway. n=3; *P<0.05 and **P<0.01 compared with miRNA-NC group. miR/miRNA, microRNA; HUVECs, human umbilical vein endothelial cells; ITGB1, integrin β1; OGT, O-linked N-acetylglucosamine transferase 110 kDa subunit; OGA, β-N-acetylglucosaminidase; TCF1, T-cell factor 1; p- phosphorylated; hsa-miR, human microRNA; NC, negative control; WT, wild-type; Mut, mutant.

on mucin structure and cell localization (20). MUC1, a transmembrane glycoprotein belonging to membrane-associated subgroup, is abnormally expressed numerous cancers and functions as a key oncogene in tumorigenesis, participating in cell regeneration, differentiation, integration and adhesion by regulating the p53 and β-catenin pathways (20,34). MUC1 has been reported to activate and regulate the Wnt/β-catenin pathway and the expression of its key downstream gene, c-Myc in intrahepatic cholangiocarcinoma (20). Therefore, it was hypothesized that in IH, the membrane proteins ITGB1 and MUC1 could promote c-Myc mRNA transcription through the β-catenin pathway. Our previous study using miRNA sequencing and protein-protein interaction analyses identified genes and miRNAs related to proteoglycan signaling pathways in angiogenesis and cancer, including c-Myc, ITGB1, Bcl2, MUC1 and miR-29a in the HUVEC cell model (11). In the previous study, miR-29a was upregulated, while c-Myc, ITGB1, Bcl2 and MUC1 were downregulated in propranolol-treated HUVECs (11). The results of the present study revealed that inhibition of MUC1 inhibited c-Myc expression and the viability of HUVECs. Meanwhile, miR-29a mimic transfection significantly inhibited the expression of the ITGB1, ITGB1-DT, MUC1, TCF1 and c-Myc genes in HUVECs. TCF1 acts as a key downstream effector of the β-catenin signaling pathway, confirming the inhibitory effect of miR-29a mimics on this pathway. However, the detailed interactions between miR-29a and MUC1 remain ambiguous, and it is not clear whether miR-29a affects the expression of MUC1 directly or indirectly; the lack of luciferase assay or target prediction analyses between miR-29a and MUC1 is a limitation of the present study. Thus, further investigations are warranted.

It was found that inhibition of OGT downregulated the c-Myc pathway in HUVECs in the present study. In addition, miR-29a mimics inhibited OGA and OGT expression, while the use of

an miR-29a inhibitor promoted OGA and OGT expression. Thus, inhibition of OGT and OGA may alleviated the progression of IH. However, the association between miR-29a and the OGT/OGA pathway remains to be fully elucidated; proteomics analysis may be required to further elucidate this association. Additionally, the absence of an *in vivo* animal study and the lack of validation using clinical IH samples limits the translation of the present findings. Therefore, the expression levels of miR-29a, OGT, OGA and ITGB1 in clinical IH samples, as well as the role of miR-29a in a hemangioma subcutaneous xenograft mouse model, should be further explored in the future.

In the present study, it was found that miR-29a served an important role in regulating the proliferation of HUVECs. In addition, miR-29a was able to regulate the β-catenin/c-Myc pathway and the viability of HUVECs by directly targeting ITGB1. Furthermore, miR-29a regulated MUC1/c-Myc signaling. Therefore, the findings of the present study may serve as a theoretical basis for the clinical research of IH and miR-29a may serve as a potential therapeutic target for IH.

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Availability of data and materials

The data generated in the present study may be requested from the corresponding author.

Authors' contributions

QS and WC were responsible for the study conception and design. WC, SZ, XZ, YW, YQ, TZ and LZ contributed towards data collection. QS, WC, LZ, TZ and WL were responsible for the analysis and interpretation of results. QS and WL were responsible for drafting the manuscript. WL supervised the entire experimental process. QS and WL confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

The use of HUVECs was approved by the ethics committee of Children's Hospital of Soochow University (approval no. 2025CS332).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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