ORIGINAL RESEARCH

miR-342-5p Is a Notch Downstream Molecule and Regulates Multiple Angiogenic Pathways Including Notch, Vascular Endothelial Growth Factor and Transforming Growth Factor β Signaling

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***Background*—**Endothelial cells (ECs) form blood vessels through angiogenesis that is regulated by coordination of vascular endothelial growth factor (VEGF), Notch, transforming growth factor β, and other signals, but the detailed molecular mechanisms remain unclear.

***Methods and Results*—**Small RNA sequencing initially identified miR-342-5p as a novel downstream molecule of Notch signaling in ECs. Reporter assay, quantitative reverse transcription polymerase chain reaction and Western blot analysis indicated that miR-342-5p targeted endoglin and modulated transforming growth factor β signaling by repressing SMAD1/5 phosphorylation in ECs. Transfection of miR-342-5p inhibited EC proliferation and lumen formation and reduced angiogenesis in vitro and in vivo, as assayed by using a fibrin beads–based sprouting assay, mouse aortic ring culture, and intravitreal injection of miR-342-5p agomir in P3 pups. Moreover, miR-342-5p promoted the migration of ECs, accompanied by reduced endothelial markers and increased mesenchymal markers, indicative of increased endothelial–mesenchymal transition. Transfection of endoglin at least partially reversed endothelial–mesenchymal transition induced by miR-342-5p. The expression of miR-342-5p was upregulated by transforming growth factor β, and inhibition of miR-342-5p attenuated the inhibitory effects of transforming growth factor β on lumen formation and sprouting by ECs. In addition, VEGF repressed miR-342-5p expression, and transfection of miR-342-5p repressed VEGFR2 and VEGFR3 expression and VEGF-triggered Akt phosphorylation in ECs. miR-342-5p repressed angiogenesis in a laser-induced choroidal neovascularization model in mice, highlighting its clinical potential.

***Conclusions*—**miR-342-5p acts as a multifunctional angiogenic repressor mediating the effects and interaction among angiogenic pathways. **(*J Am Heart Assoc*. 2016;5:e003042 doi: 10.1161/JAHA.115.003042)**

Key Words: angiogenesis • endothelial cell • endothelial cell differentiation • microRNA • Notch • vascular endothelial growth factor

Endothelial cells (ECs) lining blood vessels play pivotal roles in transporting oxygen and nutrients to tissues and taking metabolic wastes away and participate in regulating tissue homeostasis. In response to various environmental insults, quiescent ECs undergo a wide variety of functional and morphological remodeling.[1] Consequently, hypoxia induces the formation of new vessels from preexisting ones, a process defined as *angiogenesis* and characterized by coordinated sprouting, proliferation, oriented migration, and lumen formation of ECs.[2,3] Angiogenesis is essential for normal development and is involved in a large number of postnatal diseases including ischemic damages, intraocular neovascularization disorders, and cancers.[4] Moreover, in tumor microenvironments, fibrogenic tissues, and the artery wall in pulmonary artery hypertension, ECs can lose their endothelial markers, such as zonula occludens 1, and gain mesenchymal markers including fibroblast-specific protein 1 (FSP1), vimentin, β-catenin, Twist, and Snail2. This process is called endothelial–mesenchymal transition (EndMT) and might contribute to both tissue homeostasis and angiogenesis.[5]

Numerous signaling pathways are involved in endothelial plasticity under physiological and pathophysiological condi-

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tions.[1–3] Vascular endothelial growth factor (VEGF) receptors, a class of receptor tyrosine kinases, regulate vascular permeability and endothelial proliferation, migration, survival, and differentiation.[6] VEGFA is the major ligand for VEGFR and binds to VEGFR2 to trigger receptor dimerization and autophosphorylation at several tyrosine residues, which in turn activate the downstream phospholipase C-γ–protein kinase pathway, leading to activation of the c-Raf, MEK, and mitogen-activated protein kinase cascade and the phosphoinositide 3-kinase and Akt pathway.[6,7] Notch signaling is an evolutionarily highly conserved pathway mediating contact-dependent signaling between neighboring cells.[8] Notch ligands (delta-like 1, 3, and 4; jagged 1 and 2) activate sequential cleavage of Notch receptors (notch 1–4), executed by ADAM17 and γ-secretase, to release the Notch intracellular domain (NICD), which translocates into the nucleus and activates the transcription of downstream genes such as Hairy and enhancer of split (Hes) family molecules. Notch signal is essential for endothelial sprouting in angiogenesis and the maintenance of vascular homeostasis. Deficiency of RBPJ, the integrative downstream transcription factor of canonical Notch signaling, leads to excessive sprouting and malformation of vessels, whereas activation of Notch signaling restricts angiogenesis.[9,10] The transforming growth factor β (TGF-β) family of pleiotrophic cytokines, including 3 isoforms of TGF-β, activins, and bone morphogenetic proteins elicits their biological functions through 5 type 2 receptors, 7 type 1 receptors (also termed activin receptor-like kinases [ALKs]), and auxiliary receptors endoglin and betaglycan.[11] On ligand binding, TGF-β type 2 receptors phosphorylate type 1 receptors on specific serine and threonine residues in the intracellular domain, leading to the recruitment and phosphorylation of receptor-regulated Smads, which associate with Smad4 to regulate the transcription of specific target genes. In endothelial cells, TGF-β signals via both ALK1 and ALK5, which induce the phosphorylation of Smad1/5/8 and Smad2/3, respectively. Signaling of TGF-β/ALK1–Smad1/5 stimulates EC migration, proliferation, and tube formation, whereas TGF-β/ALK5–Smad2/3 signaling inhibits angiogenesis.[12] Endoglin is not directly involved in TGF-β receptor signaling but modulates signaling responses of multiple members of the TGF-β family.[13] Mutation or abnormal expression of endoglin is the etiological reason for hereditary hemorrhagic telangiectasia or preeclampsia, respectively, which are closely related to malformation and dysfunction of blood vessels.[14,15] Inhibiting endoglin by gene knockdown in ECs inhibits TGF-β/ALK1 signaling, and potentiates TGF-β/ALK5 signaling,[16–18] resulting in reduced proliferation.[17,19] In contrary, endoglin overexpression suppresses TGF-β/ALK5 signaling.[20,21] Mouse embryos lacking ALK1, ALK5, TGF-β type 2 receptor, or endoglin die during midgestation because of impaired vascular development,[12] further highlighting the significance of TGF-β signaling in the vascular system. In addition, TGF-β signaling is also the major signal driving EndMT through the major transcription factors Snail and Slug,[5] which are also modulated by Notch and other pathways.[22,23] Although each of these signaling pathways is essential for vascular development, coordinated regulation of ECs through signaling pathway interactions guarantees normal vascular morphogenesis and homeostasis.[24] Nevertheless, how these signals crosstalk remains unclear.

MicroRNAs (miRNAs) are a family of short noncoding RNAs that regulate gene expression by binding to the 3′ untranslated region (UTR) of mRNAs with seed sequence. Evidence has indicated that miRNAs are essential for vessel development and homeostasis.[25,26] Knockout of Dicer, an enzyme essential for miRNA biogenesis, specifically in ECs results in reduced angiogenic response to limb ischemia and VEGF stimulation.[27] It has been speculated that miRNAs are potential targets of therapies for cardiovascular diseases.[28] In this study, we identified miR-342-5p as a downstream molecule of Notch signaling that is involved in the regulation of ECs during angiogenesis and EndMT. miR-342-5p is encoded in the intron of the Ena-vasodilatorstimulated phosphoprotein-like (*EVL*) gene, which may function as a tumor suppressor.[29] We found that the expression of miR-342-5p and its host gene *EVL* increased during angiogenesis, and its expression in ECs could be regulated by Notch signaling and VEGF and TGF-β treatment. Overexpression of miR-342-5p in ECs attenuated angiogenesis in vitro and in vivo and promoted EndMT. Our results clarified miR-342-5p as a novel molecule that regulates both angiogenesis and EndMT, most likely through modulating VEGFR, Notch, and endoglin-mediated TGF-β signaling.

Materials and Methods

Human Tissues and Animals

Human umbilical cord biopsies were obtained from the Department of Gynecology and Obstetrics of Xijing Hospital, Fourth Military Medical University. All human participants in the study had signed informed consent for the use of their tissue samples. The protocols involving human samples were approved by the ethics committee of Xijing Hospital, Fourth Military Medical University.

ROSA-Stop^{f}-NICD mice (a gift from H.L. Li), which contain mouse Notch1 NICD (aa1749-2293 lacking PEST domain) followed by internal ribosomal entry site–linked green fluorescent protein in the GT(ROSA)26Sor locus, were mated with Cdh5-CreER mice (Jackson Laboratory, Bar Harbor, ME) to obtain endothelial-specific Notch-activating mice (NICD^{ECA}) after administration of tamoxifen. Pups of Balb/c mice at postnatal day 3 were intravitreally injected with 6 μg of

miR-342-5p agomir in 0.5 μL PBS in one eye and 6 μg of control oligonucleotides in the other. Retinas were collected on postnatal day 7, fixed in 4% paraformaldehyde at 4°C overnight, and immunostained as described below. Laser-induced choroidal neovascularization (CNV) assay was performed, as described previously.[30] Mice were anesthetized, and the pupils were dilated. Six laser burns were made at the 2, 4, 6, 8, 10, and 12 o'clock positions of the posterior pole around the optic nerve of both eyes. On the next day, mice were intravitreally injected with 3 μg of miR-342-5p agomir or control oligonucleotides in either eye. Choroid membranes were collected and stained on day 7 after laser burns. Mice used in the CNV model were handled in accordance with guidelines from the Association for Research in Vision and Ophthalmology. All animal experiments were approved and followed the guidelines issued by the Animal Experiment Administration Committee of the Fourth Military Medical University.

Cell Culture and Transfection

Primary human umbilical vein ECs (HUVECs) were cultured in an EC medium (ScienCell) supplemented with 5% FBS and EC growth supplements. Cells were used in experiments between passages 2 and 5. HeLa cells were maintained in DMEM supplemented with 10% FCS. The γ-secretase inhibitor (Alexis Biochemicals), VEGF, and TGF-β (Promega) were used at concentrations of 25, 30, and 2.5 ng/mL, respectively. Inhibitors of Akt (LY294002) and ERK (ERK inhibitor) were purchased from Cayman Chemical Company and used at the concentrations of 10 and 20 μmol/L, respectively.

HUVECs were transfected with miR-342-5p mimics or control or miR-342-5p antisense oligonucleotides (ASO) or control (RiboBio) at a concentration of 50 or 100 nmol/L by using Lipofectamin LTX reagent (Invitrogen), according to the manufacturer's instructions. The coding region of murine endoglin cDNA was cloned by polymerase chain reaction (PCR) and inserted into pcDNA3 to generate pcDNA3–mouse endoglin for transfection with Lipofectamin 2000. In some experiments, cells were transduced with lentivirus particles expressing miR-342-5p or control, which were purchased from Shanghai Genechem Co and handled according to the provider's protocols.

Cell Proliferation Assays

HUVECs were trypsinized, washed with ice-cold PBS, and fixed in 75% ethanol at 4°C for >2 hours. The fixed cells were washed again and incubated with PBS containing 0.02% Triton X-100, 3000 U/mL RNase A, and 50 μg/mL propidium iodide for 30 minutes at 37°C and analyzed by fluorescence-activated cell sorting using a FACSCalibur flow cytometer (BD Immunocytometry Systems). Data were analyzed with the ModFitLT software (Verity Software House). For staining with EdU (5-ethynyl-2′-deoxyuridine), cells were incubated with 50 μmol/L EdU (RiboBio) in medium for 2 hours and then fixed with 4% paraformaldehyde at room temperature for 30 minutes, followed by staining with Apollo 567. Images were captured under a fluorescence microscope (BX51; Olympus).

Cell Migration Assay

HUVECs were cultured to confluence. A scratch was made gently, and the medium was then replaced by EC medium supplemented with 1% FBS. Wound closure was measured at 12 hours after the scratch was made. For Transwell assay, HUVECs were trypsinized and cultivated in the Transwell chamber (Millipore) and cultured in complete medium for 12 hours. Cells migrating to the lower side of the polycarbonate membrane were stained with crystal violet and observed under a microscope.

TUNEL Assay

TUNEL (terminal dexynucleotidyltransferase-mediated dUTP nick end labeling) assay was performed using the DeadEnd Fluorometric TUNEL System (Promega), following the manufacturer's instructions. Briefly, cells were fixed with 4% paraformaldehyde and permeabilized in 0.3% Triton X-100, and then incubated with the TUNEL reagents containing cyanine 3–dUTP at 37°C for 1 hour. The samples were washed with PBS and counterstained with DAPI and observed under a fluorescence microscope.

Endothelial Lumen Formation Assay

Cultured HUVECs were trypsinized and seeded in the 48-well plates precoated with 200 μL Matrigel Basement Membrane Matrix (BD Biosciences) and incubated at 37°C for 8 hours. Images were captured under a microscope, and the number of branches and the length of cell cords in the enclosed lumen structures were determined.

Fibrin Gel Beads Sprouting Assay

A fibrin beads sprouting assay was conducted, as described previously, using a fibrin beads assay kit (Amersham-Pharmacia Biotech), according the supplier's instructions.[31] Briefly, HUVECs were incubated with the Cytodex 3 microcarrier beads (400 cells per bead; Sigma-Aldrich) at 37°C overnight. The microbeads were then embedded in the fibrinogen (Sigma-Aldrich) containing 0.625 U/mL thrombin (Sigma-Aldrich) at a density of 100 beads/mL in a 48-well plate, and 0.5 mL EGM-2

medium (Clonetics) was added with lung fibroblasts (20 000 cells per well), as described earlier. The medium was changed every other day for 2 or 4 days. Images of the beads were captured under a microscope (CKX41; Olympus) with a CCD camera (DP70; Olympus), and sprouting was quantified by measuring the number and length of sprouts.

Aortic Ring Culture

Mice (aged 8–12 weeks) were anesthetized and aortas were removed, cleaned, and transfected with 200 nmol/L miR-342-5p mimics or control oligonucleotides for 12 hours by using Lipofectamine 2000, as described earlier. The aortas were then embedded in 50 μL Matrigel Basement Membrane Matrix in a 96-well plate containing 150 μL of opti-MEM supplemented with 2.5% FBS and 30 ng/mL VEGF (Promega) and cultured at 37°C in 5% CO_2/95% air. Sproutlike structures were allowed to grow over 4 days. Thereafter, the samples were photographed under a microscope, and the number and length of sprouts were measured.

Western Blot Analysis

Cell lysates were prepared with RIPA buffer (Beyotime). Protein concentration was determined using a BCA Protein Assay kit (Pierce). Samples were separated by SDS-PAGE, blotted onto polyvinylidene fluoride membranes, and probed with primary antibodies, followed by horseradish peroxidase–conjugated goat antirabbit IgG or goat antimouse IgG (Boster Bio Tec). β-actin was used as a loading control. The primary antibodies included rabbit antihuman EVL (1:50; Santa Cruz Biotechnology), mouse antihuman endoglin (1:800; BD Biosciences), rabbit anti–phosphorylated Smad1/5 (1:1000; Cell Signaling), rabbit anti–phosphorylated Smad2/3 (1:500; Santa Cruz Biotechnology), rabbit anti–phosphorylated Akt (Ser 473, 1:800; Cell Signaling), rabbit anti-Akt (1:800; Cell Signaling), rabbit anti–phosphorylated ERK (1:1000; Cell Signaling), rabbit anti-ERK (1:1000; Cell Signaling), mouse anti-β-actin (1:1000; Sigma-Aldrich), mouse anti-CD31 antibody (1:1000; Abcam), rabbit anti–α-smooth muscle actin antibody (1:200; Abcam), mouse anti–β-catenin antibody (1:1000; Millipore), rabbit anti-vimentin antibody (1:1000; Abcam). Membranes were developed using an enhanced chemoluminescence system (Clinx Science Instruments).

Small RNA Sequencing and miRNA Profiling

Primary murine liver sinusoid endothelial cells were isolated using a recommended protocol[32] from 3 pairs of $NICD^{ECA}$ and control mice. MiRNA expression profiling by small RNA sequencing and data analysis were conducted by a commercial service (RiboBio). Briefly, total RNA was prepared from cells, and 3′ and 5′ adaptors were added to each end, respectively, followed by reverse transcription (RT) and PCR amplification. cDNA fragments derived from 18- to 30-nucleotide RNA molecules were purified by electrophoresis and sequenced on an Illumina HiSeq 2500 sequencer. Data were processed and validated routinely. Differential expression of miRNA between the 2 groups was analyzed using a cluster analysis and expressed as a scatter diagram.

Quantitative RT-PCR

Total cellular or tissue RNA was extracted with the TRIzol reagent (Invitrogen) according to the standard procedures. cDNA was synthesized using an RT kit (Takara). Real-time PCR was performed using a SYBR Premix Ex Taq Kit (Takara) and an ABI PRISM 7500 real-time PCR system (Life Technologies), with GAPDH or β-actin as internal controls. MiRNA level was quantitatively determined using a real-time RT-PCR kit, with U6 RNA as an internal control. The PCR primers are listed in Table.

Reporter Assay

The 3′ UTR of the human endoglin mRNA (NM_001114753.2, region 2415-3044) was amplified from cDNA derived from total RNA of HUVECs and subcloned into pGL3 promoter (Promega) to construct pGL3-endoglin wild type. A reporter, pGL3-endoglin-mt, with a mutation in the 3′ UTR complementary to the seed sequence of miR-342-5p was generated by PCR. HeLa cells were cotransfected with 100 ng of pGL3 promoter, pGL3-endoglin wild type, or pGL3-endoglin-mt, together with miR-342-5p mimics (25 and 50 nmol/L) and pRL-TK (5 ng). Cells were harvested 24 hours after the transfection, and firefly and renilla luciferase activities were analyzed with the Dual-Luiferase Reporter Assay System (Promega), according to the manufacturer's instruction.

Immunofluorescence

Retina or choroid membrane samples were fixed overnight at 4°C with 4% paraformaldehyde, blocked, and permeabilized in PBS containing 1% BSA and 0.3% Triton X-100, then immunostained with FITC-labeled GSL I-isolectin B4 (Vector Laboratories) or rabbit anti-Ki67 antibody (Millipore) at 4°C overnight. After washing, the samples were incubated with a cyanine 3–conjugated goat antirabbit IgG secondary antibody (Sigma-Aldrich). Images were captured with a fluorescence or confocal microscope.

Statistics

Statistical evaluation was performed with the Image-Pro Plus 6.0 and GraphPad Prism5 software. The Student *t* test was

Table. Sequences of Primers and Oligonucleotides Used in the Study

Primer Name	Sequence
miR-342-5p (qPCR)	5′-CGGAGGGGTGCTATCTGTGATTGAG -3′
U6 (qPCR)	5′-GGATGACACGCAAATTCGTGAAGC -3′
Mouse *evl* forward (qPCR)	5′-AACGGAAGACCCTAGCACCT -3′
Mouse *evl* reverse (qPCR)	5′-CTGAAGGGGGCTCTTAGCTT -3′
Mouse *endoglin* forward (qPCR)	5′-CCCTCTGCCCATTACCCTG -3′
Mouse *endoglin* reverse (qPCR)	5′-GTAAACGTCACCTCACCCCTT -3′
Mouse *cd31* forward (qPCR)	5′-ACGCTGGTGCTCTATGCAAG -3′
Mouse α-*SMA* forward (qPCR)	5′-GTCCCAGACATCAGGGAGTAA-3′
Mouse α-*SMA* reverse (qPCR)	5′-TCGGATACTTCAGCGTCAGGA-3′
Mouse *cd31* reverse (qPCR)	5′-TCAGTTGCTGCCCATTCATCA -3′
Human *evl* forward (qPCR)	5′-CACGACGAGAGCTCCATGTCA -3′
Human *evl* reverse (qPCR)	5′-TGGCCAGCAGTTTGTTCATTTC -3′
Human *endoglin* forward (qPCR)	5′-CGCCAACCACAACATGCAG -3′
Human *endoglin* reverse (qPCR)	5′-GCTCCACGAAGGATGCCAC -3′
Human *zo-1* forward (qPCR)	5′-ACCAGTAAGTCGTCCTGATCC -3′
Human*zo-1* reverse (qPCR)	5′-TCGGCCAAATCTTCTCACTCC -3′
Human *fsp1* forward (qPCR)	5′-TTCGGGAATGTGACTACAAC -3′
Human *fsp1* reverse (qPCR)	5′-ATGACAGCAGTCAGGATCTG -3′
Human *snail2* (*slug*) forward (qPCR)	5′-TTTCCAGACCCTGGTTGCTTC -3′
Human *snail2* (*slug*) reverse (qPCR)	5′-CTCAGATTTGACCTGTCTGCAAATG -3′
Human *twist* forward (qPCR)	5′-GGAGTCCGCAGTCTTACGAG -3′
Human *twist* reverse (qPCR)	5′-TCTGGAGGACCTGGTAGAGG -3′
Human β-*catenin* forward (qPCR)	5′-ATTTGATGGAGTTGGACATGG -3′
Human β-*catenin* reverse (qPCR)	5′-TGTTCTTGAGTGAAGGACTGA -3′
Human *vimentin forward* (qPCR)	5′-GAGAACTTTGCCGTTGAAGC -3′
Human *vimentin* reverse (qPCR)	5′-GCTTCCTGTAGGTGGCAATC -3′
Human *cd31* forward (qPCR)	5′-AACAGTGTTGACATGAAGAGCC -3′
Human *cd31* reverse (qPCR)	5′-TGTAAAACAGCACGTCATCCTT -3′
Human *gapdh* forward (qPCR)	5′-GCACCGTCAAGGCTGAGAAC -3′
Human *gapdh* reverse (qPCR)	5′-TGGTGAAGACGCCAGTGGA -3′
Mouse β-*actin* forward (qPCR)	5′-CATCCGTAAAGACCTCTATGCCAAC -3′
Mouse β-*actin* reverse (qPCR)	5′-ATGGAGCCACCGATCCACA -3′
Human *endoglin* 3′-UTR forward (clone)	5′-GAATTCTCGCCCAGCAGGAGAGAC -3′
Human *endoglin* 3′-UTR reverse (clone)	5′-CTGCAGTTGGTGGTGAATACACAGGGG -3′
Human *endoglin* 3′-UTR mt forward (clone)	5′-GTCCCTCTCACTAACAGAACCTTC -3′
Human *endoglin* 3′-UTR mt reverse (clone)	5′-GAAGGTTCTGTTAGTGAGAGGAC -3′

qPCR indicates quantitative polymerase chain reaction.

used for statistical analyses (unless otherwise specified). Data were expressed as mean±SD. $P<0.05$ was considered statistically significant.

Results

miR-342-5p Was Downstream to Notch Signaling in ECs and Associated With Angiogenesis

In an attempt to identify downstream targets of Notch signaling, we isolated liver sinusoid endothelial cells from transgenic mice with EC-specific conditional expression of a constitutively active NICD ($NICD^{ECA}$) and control mice and screened differentially expressed miRNAs by using small RNA sequencing (Figure 1A). Among a group of differentially expressed miRNAs, miR-342-5p emerged as a potential molecule regulated by Notch signaling because it also exhibited differential expression in other cell types when Notch signal was interfered (unpublished data). In mouse liver sinusoid endothelial cells isolated from $NICD^{ECA}$ mice, RT-PCR further confirmed upregulation of miR-342-5p and *EVL* that harbor the miR-342-5p gene in both mouse and human (Figure 1B). Conversely, in HUVECs treated with γ-secretase inhibitor, the expression of both miR-342-5p and EVL mRNA was downregulated (Figure 1C), suggesting that miR-342-5p was a novel downstream molecule of Notch signaling.

To determine whether miR-342-5p was differentially regulated during angiogenesis, we cultured HUVECs under proliferative conditions and in lumen structures and compared the expression of miR-342-5p and *EVL* using quantitative RT-PCR. The results indicated that miR-342-5p expression increased almost 2-fold in ECs in lumen structures (Figure 1D). We isolated retinal vasculature from adult mice and P3 pups that was undergoing vigorous angiogenesis[33] and compared expression of miR-342-5p and *EVL*. The result showed that the expression of miR-342-5p and EVL mRNA decreased concomitantly in adult retinal vasculature (Figure 1E). These results suggested that the expression of miR-342-5p and its host gene was dynamically regulated in ECs during angiogenesis.

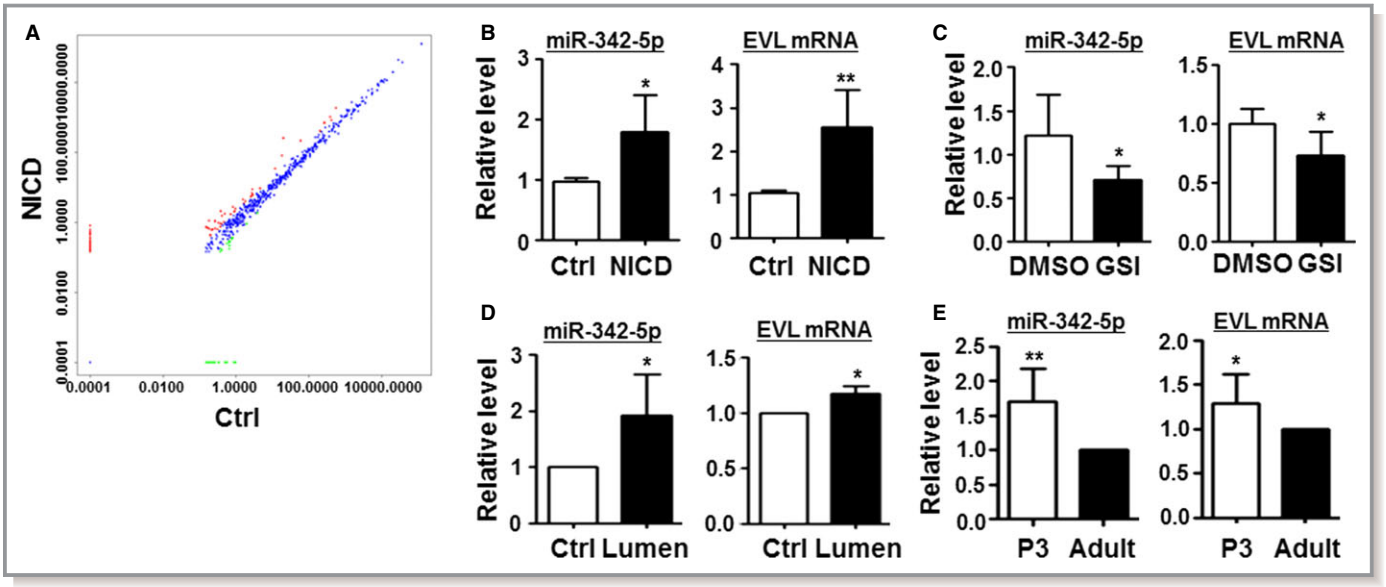

Figure 1. miR-342-5p was a Notch downstream molecule involved in angiogenesis. A, LSECs were isolated from $NICD^{ECA}$ and control mice and subjected to microRNA profiling. B, LSECs were isolated from $NICD^{ECA}$ and control mice, and expression of miR-342-5p and *EVL* mRNA was determined by qRT-PCR. C, HUVECs were cultured in the presence of γ-secretase inhibitor or dimethyl sulfoxide, and the expression of miR-342-5p and EVL mRNA was determined by using qRT-PCR. D, HUVECs were cultured under proliferative conditions and were induced to form lumen structures. The expression of miR-342-5p and *EVL* mRNA was compared for proliferative HUVECs and HUVECs in lumen structures. E, Retinal vessels were collected from P3 pups and adult mice. The expression of miR-342-5p and EVL mRNA was determined with qRT-PCR. Bars indicate mean±SD (n=6), $*P<0.05$, $**P<0.01$. Ctrl indicates control; HUVEC, human umbilical vein endothelial cells; LSEC, liver sinusoid endothelial cell; NICD, Notch intracellular domain; $NICD^{ECA}$, endothelial-specific Notch-activating mice; P3, postnatal day 3; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

Endoglin mRNA Was a Direct Target of miR-342-5p

The sequence of 3′ UTR of endoglin mRNA appeared to be recognizable by the seed region of miR-342-5p (Figure 2A). Indeed, transfection with miR-342-5p reduced endoglin protein level, and transfection of miR-342-5p ASO increased endoglin protein level (Figure 2B and 2C). In HUVECs transfected with miR-342-5p and newborn mouse retinas that had been injected with miR-342-5p agomir, the endoglin mRNA level was also downregulated (Figure 2D). Reporter assay indicated that miR-342-5p significantly inhibited the expression of the luciferase gene containing the 3′ UTR of endoglin, and this inhibitory effect was canceled when the miR-342-5p recognition site was disrupted by mutation (Figure 2D). These results suggested that the endoglin mRNA was an authentic target of miR-342-5p. In line with this conclusion, forced Notch activation (which upregulated miR-342-5p) in ECs reduced endoglin expression (Figure 2E).

Endoglin is a coreceptor of TGF-β receptor signaling pathway and can enhance the phosphorylation of Smad1/5 to promote EC proliferation, migration, and lumen formation. In HUVECs transfected with miR-342-5p mimics, the phosphorylation of Smad1/5 decreased apparently after TGF-β treatment, whereas the phosphorylation of Smad2/3 remained unchanged (Figure 2F). Endoglin facilitates Akt phosphorylation. We found that miR-342-5p reduced Akt phosphorylation, which was rescued by overexpression of endoglin (Figure 2G). This suggested that miR-342-5p could function as a negative regulator of TGF-β signaling in ECs.

miR-342-5p Suppressed ECs Proliferation and Lumen Formation In Vitro

We next examined the effect of miR-342-5p overexpression on ECs. HUVECs were transfected with miR-342-5p or control. As shown in Figure 3A, miR-342-5p overexpression significantly reduced the number of cells in the synthesis, gap 2, and mitosis phases of cell cycle. This was confirmed by the assay that incorporated EdU and indicated that transfection of the miR-342-5p reduced EdU-positive HUVECs in culture (Figure 3B). These data suggested that overexpression of miR-342-5p in ECs inhibited cell proliferation in vitro. miR-342-5p overexpression did not alter apoptosis in HUVECs (data not shown).

We examined the effect of miR-342-5p on lumen formation by ECs in vitro. HUVECs were transfected with miR-342-5p or control for 48 hours. The result showed that miR-342-5p overexpression inhibited lumen formation by ECs (Figure 3C). In contrast, lumen formation increased by HUVECs that had been transfected with miR-342-5p ASO (Figure 3C). These

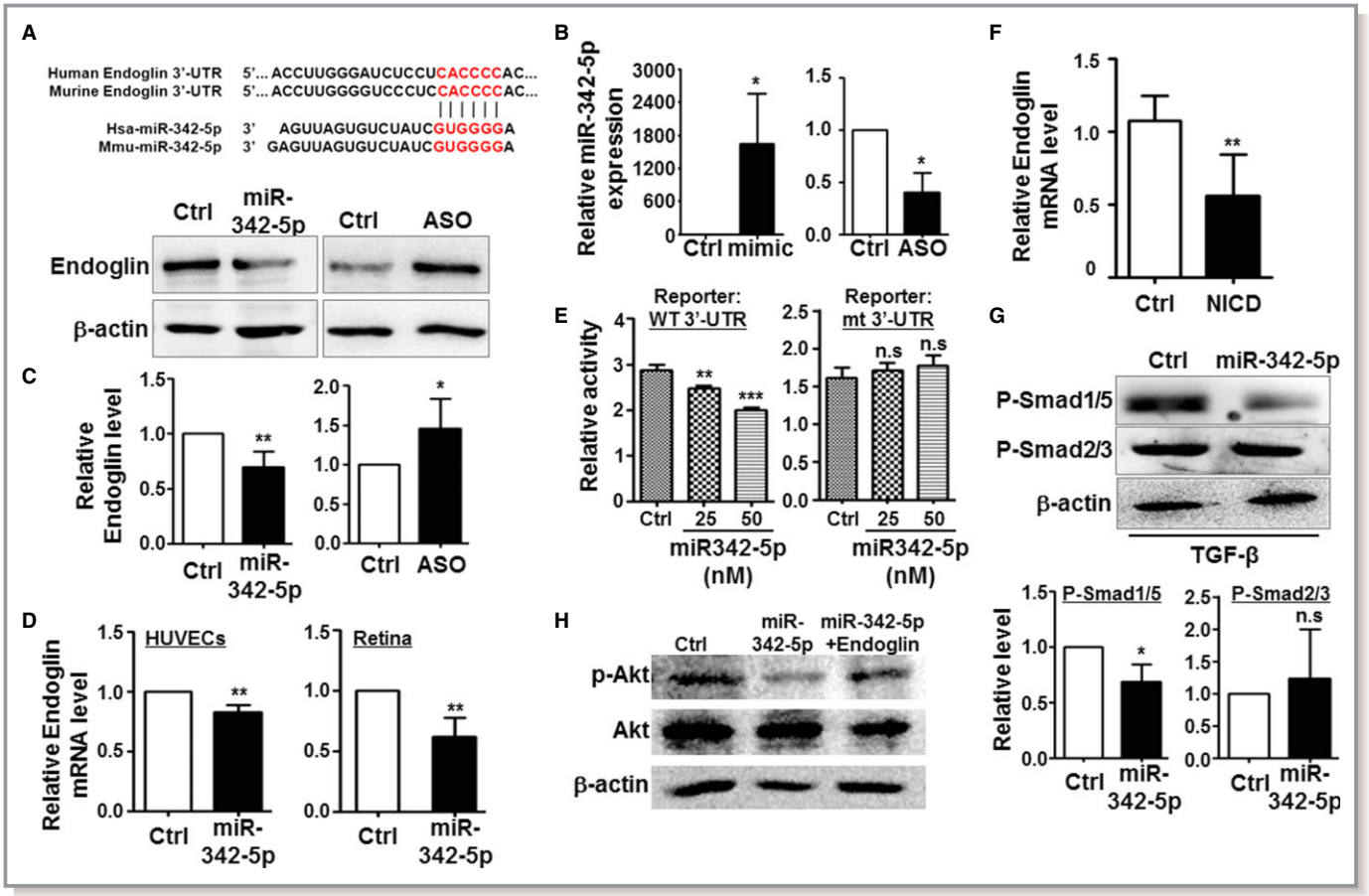

Figure 2. Identification of endoglin as a direct target of miR-342-5p. A, The 3′ UTR of human and mouse endoglin mRNA were aligned with Hsa-miR-342-5p and Mmu-miR-342-5p. Complementary sequences were marked in red. B and C, HUVECs were transfected with miR-342-5p, miR-342-5p ASO, or control. The level of miR-342-5p was determined with qRT-PCR (B). Endoglin expression was determined by using Western blot (C). Bands were quantitatively compared between groups. D, Total RNA was prepared from HUVECs transfected with miR-342-5p or retina tissues derived from pups at postnatal day 7 that had accepted intravitreal injection of miR-342-5p on postnatal day 3. Endoglin mRNA level was determined by using qRT-PCR. E, Reporter assay. HeLa cells were cotransfected with pGL3-endoglin wild type or pGL3-endoglin mutant, together with increasing amount of miR-342-5p. Cells were harvested 24 hours after the transfection, and luciferase activity was analyzed. F, Liver sinusoid endothelial cells were isolated from endothelial-specific Notch-activating mice and control mice, and expression of endoglin was determined by using qRT-PCR. G, HUVECs transfected with miR-342-5p or control were cultured in the presence of TGF-β for 30 minutes. The phosphorylation of Smad1/5 and Smad2/3 was determined by using Western blot and quantitatively compared. H, HUVECs were transfected with control, miR-342-5p or miR-342-5p plus endoglin and stimulated with vascular endothelial growth factor. The total and phosphorylated Akt were determined using Western blot. Bars indicate mean±SD (n=5), *P<0.05, **P<0.01, ***P<0.001. ASO indicates antisense oligonucleotides; Ctrl, control; HUVEC, human umbilical vein endothelial cells; Hsa, Homo Sapiens; LSEC, liver sinusoid endothelial cell; n.s., not significant; Mmu, Mus musculus; qRT-PCR, quantitative reverse transcription polymerase chain reaction; TGF-β, transforming growth factor β; UTR, untranslated region.

results suggested that miR-342-5p repressed lumen formation by ECs in vitro.

miR-342-5p Inhibited Angiogenic Sprouting In Vitro and In Vivo

HUVECs were transfected with miR-342-5p and subjected to the fibrin gel beads assay. The result showed that in the miR-342-5p–overexpressing group, the length of sprouts reduced significantly (Figure 4A, upper). We further transduced HUVECs with a lentivirus expressing miR-342-5p and green fluorescent protein, with lentivirus expressing green fluorescent protein only as a control (Figure 4B, left). The transduced cells were mixed with untransduced HUVECs at a ratio of 1:1 and were tested by using the fibrin gel beads assay. The result showed that the percentage of HUVECs overexpressing miR-342-5p appeared to have normal or slightly increased opportunity to locate at the sprouting tips compared with the control (Figure 4A, lower). We also estimated the effect of miR-342-5p on angiogenesis by culturing mouse aortic rings transfected with the miR-342-5p mimics or the control (Figure 4B, right), and the result confirmed that the overex-

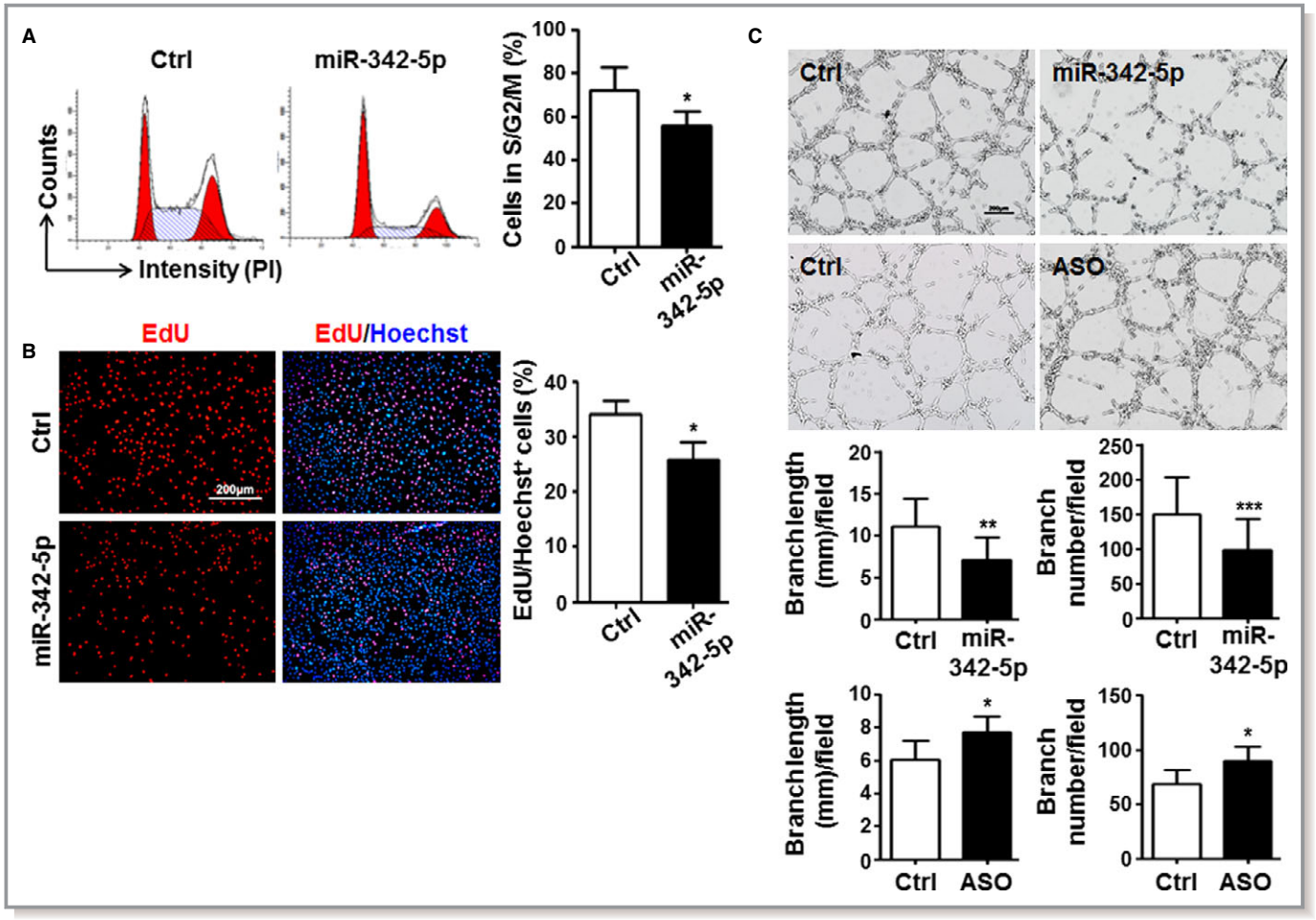

Figure 3. miR-342-5p suppressed endothelial cell proliferation and lumen formation in vitro. A, HUVECs were transfected with miR-342-5p or control. Cell cycle progression was analyzed by using fluorescence-activated cell sorting 48 hours after the transfection and compared between the 2 groups. B, HUVECs were transfected as in (A), and EdU incorporation was determined under a fluorescence microscope and quantitatively compared. C, HUVECs were transfected with miR-342-5p, miR-342-5p ASO, or control and tested for lumen formation. The number of branches and the length of cell cords of the enclosed lumens were measured. Bars indicate mean±SD (n=5), $*P<0.05$, $**P<0.01$, $***P<0.001$. ASO indicates antisense oligonucleotides; Ctrl, control; HUVEC, human umbilical vein endothelial cells; EdU, 5-ethynyl-2′-deoxyuridine; PI, propidium iodide. S/G2/M, synthesis, gap 2, and mitosis.

pression of miR-342-5p delayed the growth of angiogenic sprouts (Figure 4C). Consequently, miR-342-5p could inhibit angiogenic sprouting in vitro.

We then assessed the role of miR-342-5p in angiogenesis in vivo. Pups at postnatal day 3 were intravitreally injected with miR-342-5p agomir or the control. The retinas were collected on postnatal day 7, followed by whole-mount staining with isolectin B4 to elicit the retinal vasculature. Compared with the control group, miR-342-5p agomir-treated mice displayed smaller vessel area and fewer enclosed vessels (Figure 4D). Consistent with the in vitro data, $Ki67^+$ ECs in the retina decreased significantly in the miR-342-5p–injected group (Figure 4D). These results suggested that miR-342-5p repressed angiogenesis in vivo. This could be related to increased Dll4 and Hey1 and decreased jagged 1 expression in HUVECs transfected with miR-342-5p (Figure 4E).

miR-342-5p Promoted EndMT

Endoglin was reported as an inhibitor of EndMT in ECs and the deficiency of endoglin in ECs could promote EndMT in tumor vasculature.[34] We examined whether miR-342-5p might promote EndMT. HUVECs were transfected with miR-342-5p and the control. In both the cell scratch assay and the Transwell assay, HUVECs overexpressing miR-342-5p showed enhanced cell migration (Figure 5A and 5B, upper). In contrast, transfection with the miR-342-5p ASO reduced HUVEC migration in vitro (Figure 5A). Under microscope, miR-342-5p exhibited mesenchymal morphology and expressed both endothelial (CD31) and mesenchymal (α-smooth muscle actin) markers (Figure 5B, middle and lower). The expression of EndMT-associated genes was examined by using quantitative RT-PCR and Western blot. The result showed that

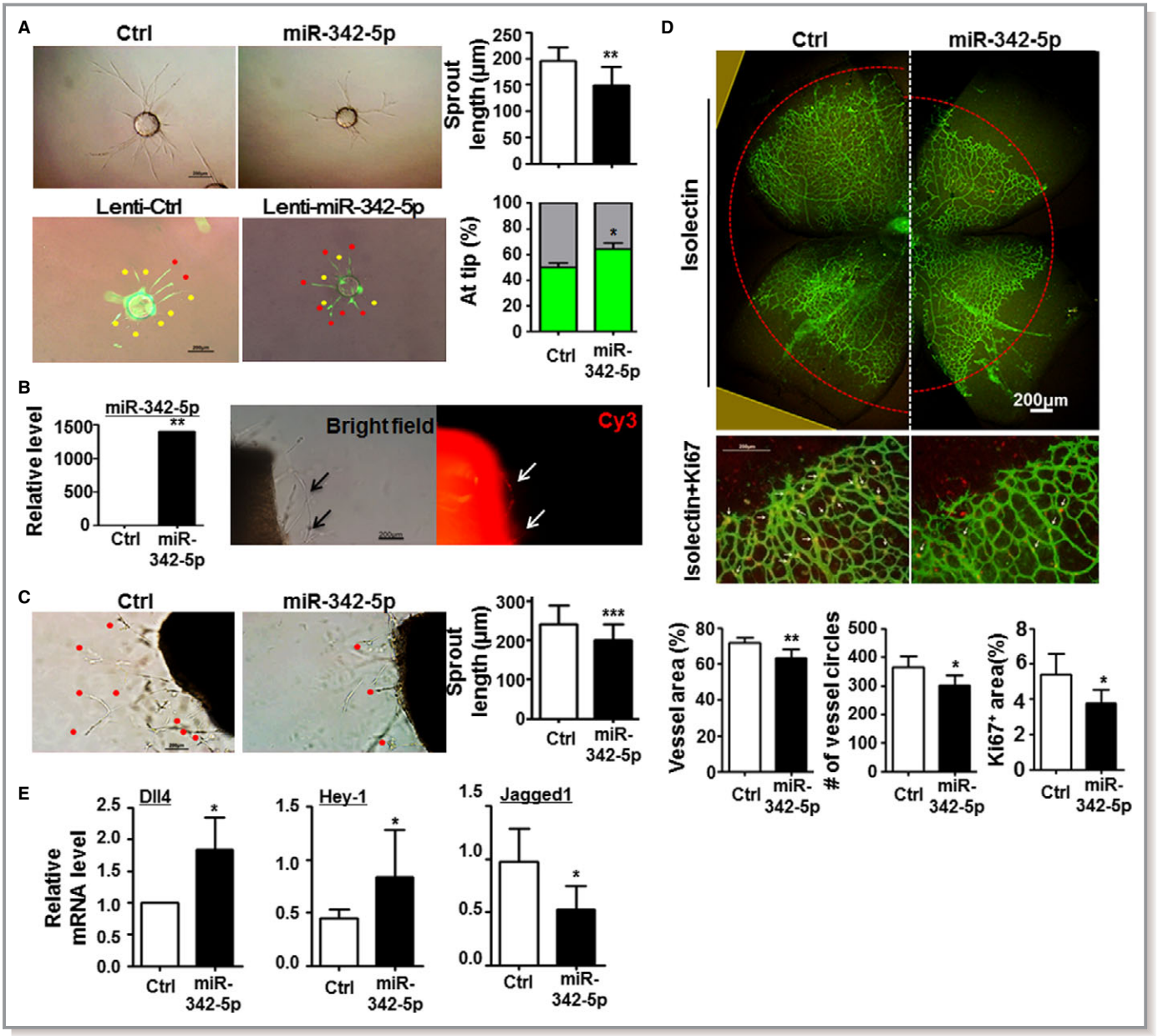

Figure 4. miR-342-5p suppressed angiogenesis. A, HUVECs were transfected with miR-342-5p or control (upper), or transduced with a lentivirus overexpressing miR-342-5p and EGFP (lower). Cells were trypsinized 24 hours after the transfection and tested for sprouting with the fibrin gel beads assay. Images were captured under a microscope, and sprouting was quantitatively analyzed by measuring the length of sprouts (upper) and the percentage of transfected cells ($EGFP^+$) on tip (lower). B, Overexpression of miR-342-5p in HUVECs or cultured aortic rings: (left) HUVECs were transduced with miR-342-5p lentivirus or control, and the level of miR-342-5p was determined with qRT-PCR; (right) cultured aortic rings were transfected with the control oligonucleotides of miR-342-5p labeled with Cy3. Pictures were taken 24 hours after the transfection. C, Aortic rings isolated from normal mice were transfected with miR-342-5p or control and cultured for 4 days in Matrigel Basement Membrane Matrix containing vascular endothelial growth factor. Images were captured and sprout length was compared between the 2 groups. D, Pups at postnatal day 3 were injected intravitreally with miR-342-5p agomir or control. Retinas were collected on postnatal day 7, stained with isolectin B4 or isolectin B4 plus anti-Ki67 and photographed under a fluorescence microscope. The percentage of vessel area and the number of enclosed vessels were quantitatively compared between the 2 groups. E, HUVECs were transfected with miR-342-5p or control. The expression of Dll4, Hey1 and jagged 1 was determined using qRT-PCR. Bars indicate mean±SD (n=5), *P<0.05, **P<0.01, ***P<0.001. Ctrl indicates control; Cy3, cyanine 3; EGFP, enhanced green fluorescent protein; HUVEC, human umbilical vein endothelial cells; Lenti, lentivirus; qRT-PCR, quantitative reverse transcription polymerase chain reaction.

overexpression of miR-342-5p downregulated endothelial-related genes such as CD31 and zonula occludens 1, whereas the expression of mesenchymal-related genes including FSP1, vimentin, Twist, β-catenin, and Snail2 (Slug) was upregulated significantly (Figure 5C and 5D). Intravitreal injection of miR-342-5p also reduced CD31 and increased α-smooth muscle

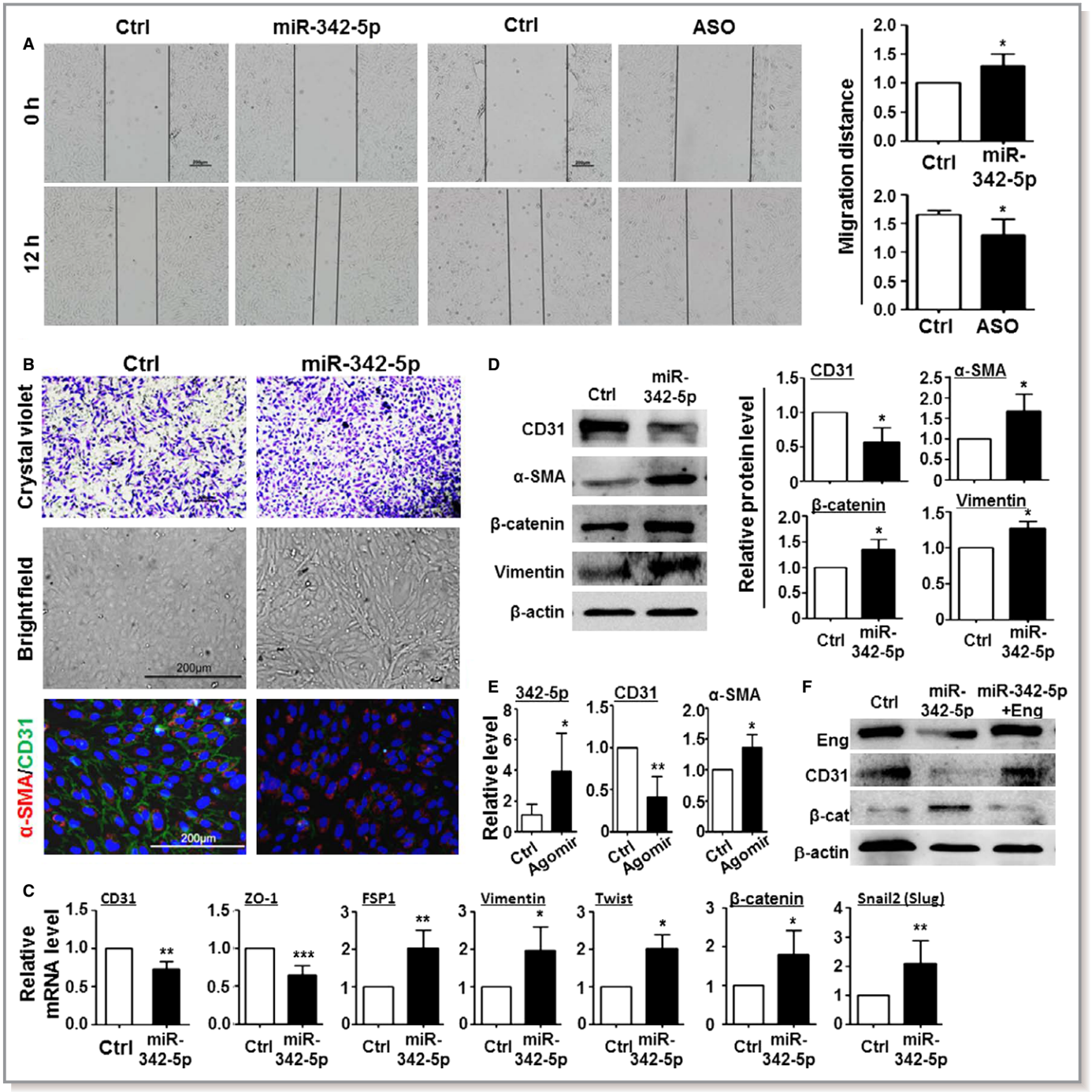

Figure 5. miR-342-5p promoted endothelial–mesenchymal transition in vitro. A, HUVECs were transfected with miR-342-5p, miR-342-5p ASO, or control. Cell migration was determined 24 hours after the transfection using a scratch assay. B, HUVECs were transfected with miR-342-5p or control: (upper) Cell migration was determined 24 hours after the transfection using a Transwell assay; (middle and lower) cells were observed under bright field or fluorescence microscope after staining with anti–α-SMA and anti-CD31. C, HUVECs were transfected with miR-342-5p or control. The expression of CD31, ZO-1, FSP1, vimentin, Twist, β-catenin, and Snail2 was determined 72 hours after transfection using qRT-PCR. D, HUVECs were transfected with miR-342-5p or control. The expression of CD31, α-SMA, β-catenin, and vimentin was determined 48 hours after the transfection using Western blot. E, Healthy pups at postnatal day 3 were injected intravitreally with miR-342-5p agomir. The level of miR-342-5p, CD31, and α-SMA was determined on postnatal day 7 using qRT-PCR. F, HUVECs were transfected with control, miR-342-5p, or miR-342-5p plus endoglin. The expression of endoglin, CD31, and β-catenin was determined 72 hours after transfection with Western blot. Bars indicate mean±SD (n=5), **P*<0.05, ***P*<0.01, ****P*<0.001. α-SMA indicates α–smooth muscle actin; β-cat, β-catenin; ASO, antisense oligonucleotides; Ctrl, control; Eng, endoglin; FSP, fibroblast-specific protein 1; HUVEC, human umbilical vein endothelial cells; qRT-PCR, quantitative reverse transcription polymerase chain reaction; ZO-1, zonula occludens 1.

actin expression (Figure 5E). Overexpression of endoglin reversed downregulation of CD31 and upregulation of β-catenin in HUVECs (Figure 5F) induced by miR-342-5p. Collectively, these data indicated that miR-342-5p could promote EndMT, likely by downregulating endoglin.

miR-342-5p Negatively Regulated TGF-β Signaling in ECs

We estimated the influence of miR-342-5p on TGF-β signaling. HUVECs were cultured in the presence of TGF-β. We found that treatment of HUVECs with TGF-β upregulated the expression of both miR-342-5p and EVL mRNA (Figure 6A). Meanwhile, TGF-β treatment reduced lumen formation and in vitro sprouting of HUVECs, which were reversed by the transfection with miR-342-5p ASO (Figure 6B and 6C). The downregulation of CD31 and upregulation of α–smooth muscle actin, β-catenin, and vimentin by TGF-β were also reversed by miR-342-5p ASO (Figure 6D). These results suggested that miR-342-5p negatively regulated TGF-β signaling.

miR-342-5p Attenuated VEGFR2 Signaling in ECs

HUVECs were treated with VEGF. RT-PCR showed that the expression of miR-342-5p and EVL decreased obviously (Figure 7A). The protein level of EVL also decreased in HUVECs on VEGF treatment (Figure 7B). Blocking VEGFR2 signaling using different downstream signaling inhibitors suggested that the expression of miR-342-5p was rescued by the Akt inhibitor

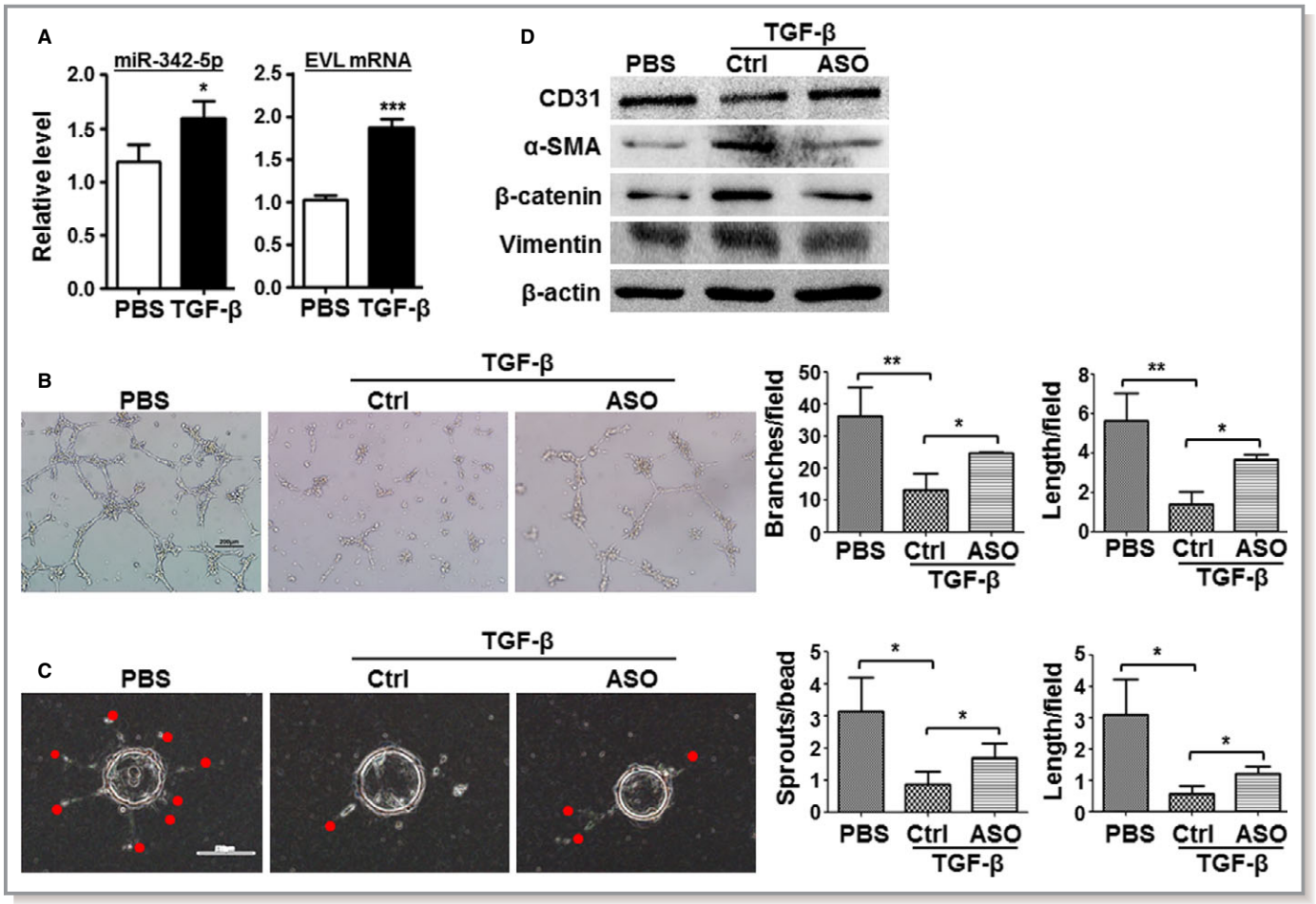

Figure 6. miR-342-5p antagonized TGF-β in endothelial cells. A, HUVECs were cultured in the presence of TGF-β or PBS, and the expression of miR-342-5p and *EVL* mRNA was determined using quantitative reverse transcription polymerase chain reaction. B, HUVECs were transfected with miR-342-5p ASO or control and cultured in the presence of TGF-β or PBS. Cells were trypsinized 72 hours after transfection and tested for lumen formation, and the number of branches and the length ($\times 10^3$ μm) of cell cords of the enclosed lumens were measured. C, Cells in (B) were tested for sprouting by using the fibrin gel beads assay. Images were captured under a microscope and sprouting was quantitatively analyzed by measuring the number and length ($\times 10^2$ μm) of sprouts. D, Cells in (B) were assayed for the protein levels of CD31, α-SMA, β-catenin, and vimentin 72 hours after the transfection by using Western blot. Bars indicate mean±SD (n=3), **P*<0.05, ***P*<0.01, ****P*<0.001. α-SMA indicates α–smooth muscle actin; ASO, antisense oligonucleotides; Ctrl, control; HUVEC, human umbilical vein endothelial cells; TGF-β, transforming growth factor β.

but not by the ERK inhibitor (Figure 7C). Moreover, consistent with the downregulation of miR-342-5p, VEGF treatment upregulated endoglin expression in HUVECs (Figure 7D).

To test whether miR-342-5p could influence VEGFR signaling, we transfected HUVECs with miR-342-5p and the control. Transfection of miR-342-5p repressed the expression of VEGFR2 and VEGFR3 (Figure 7E). The VEGFR pathway regulates EC proliferation and migration primarily through the activation of Akt and ERK signaling pathways. In HUVECs treated with VEGF, the phosphorylation of Akt and ERK1/2 increased, and transfection with miR-342-5p reduced the phosphorylation of Akt after VEGF treatment for 15 minutes (Figure 7F). These results suggested that miR-342-5p could inhibit VEGFR signaling.

miR-342-5p Ameliorated Laser-Induced CNV in Mice

The data presented suggested that miR-342-5p was a repressor of angiogenesis associated with suppressing VEGF and TGF-β signals and promoting the Notch signal. We then tested whether miR-342-5p could repress pathological angiogenesis in adult mice using the laser-induced CNV model. The results showed that the expression of miR-342-5p was upregulated significantly on days 3 and 7 after laser-mediated induction of CNV (Figure 8A), consistent with increased angiogenesis.[35] Furthermore, adult mice were subjected to laser photocoagulation-induced CNV and treated with miR-342-5p agomir injected intravitreally (Figure 8B). Choroid membranes were collected on day 7 and stained with isolectin B4. Compared with the control group, mice treated with miR-342-5p agomir showed a significant decrease in CNV area (Figure 8C). Consequently, miR-342-5p could ameliorate pathological angiogenesis in CNV.

Discussion

The present investigation revealed that miR-342-5p expression correlated with angiogenesis, and overexpression of miR-342-5p repressed EC proliferation and angiogenic sprouting, suggesting that it was a negative regulator of angiogenesis and might play a role in self-regulation of signaling pathways

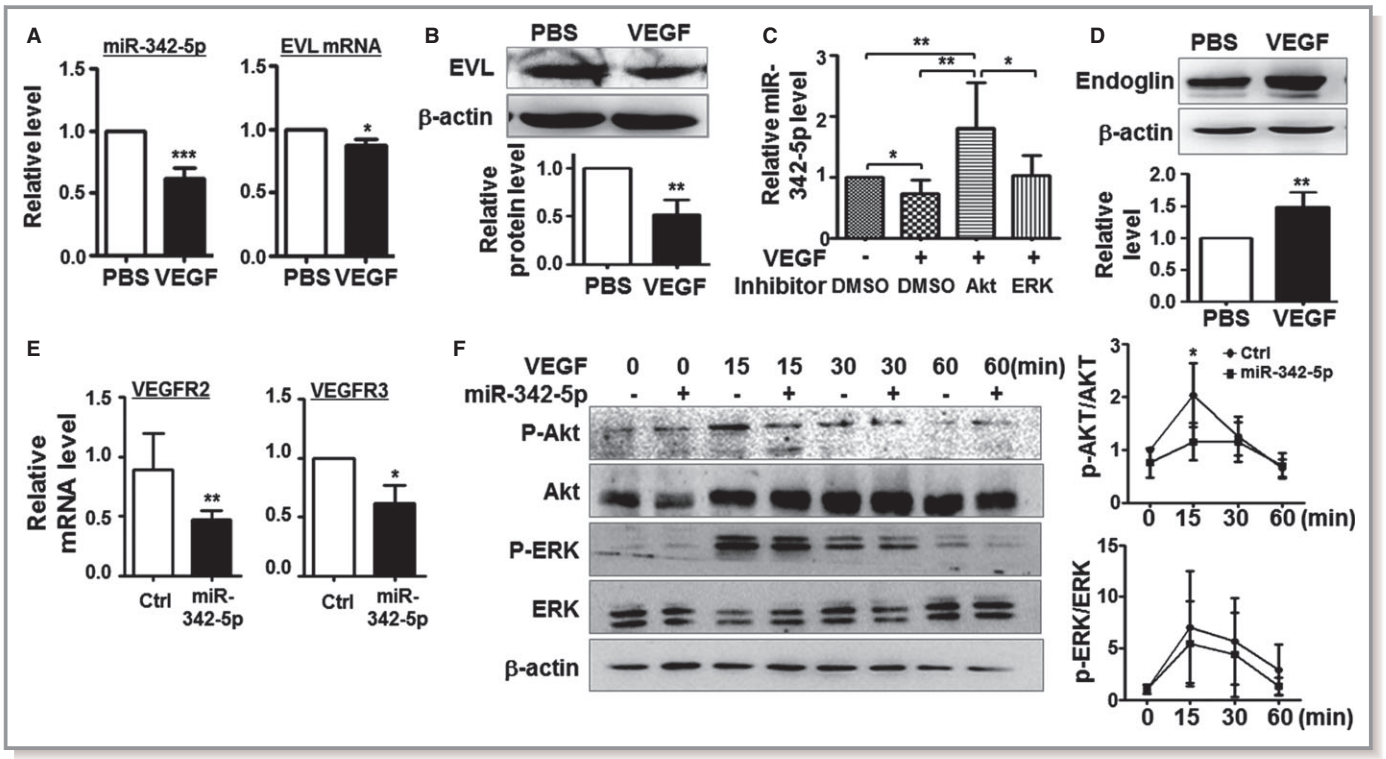

Figure 7. miR-342-5p participated in the regulation of VEGFR signaling. A and B, HUVECs were cultured in the presence of VEGF or PBS, and the expression of miR-342-5p and EVL was determined by using qRT-PCR (A) or Western blot (B). C, HUVECs were cultured with VEGF in the presence of Akt inhibitor or ERK inhibitor, and the expression of miR-342-5p and *EVL* mRNA was determined by using qRT-PCR. D, HUVECs were treated with VEGF or PBS for 48 hours. Endoglin expression was determined by using Western blot. E, HUVECs were transfected with miR-342-5p or control. The expression of VEGFR2 and VEGFR3 was determined by using qRT-PCR. F, HUVECs were transfected with miR-342-5p or control. Cells were stimulated with VEGF, and the total and phosphorylated Akt and ERK were determined by using Western blot. Bands were quantitatively compared between the 2 groups. Bars indicate mean±SD (n=6), **P*<0.05, ***P*<0.01, ****P*<0.001. Ctrl indicates control; DMSO, dimethyl sulfoxide; HUVEC, human umbilical vein endothelial cells; qRT-PCR, quantitative reverse transcription polymerase chain reaction; VEGF, vascular endothelial growth factor.

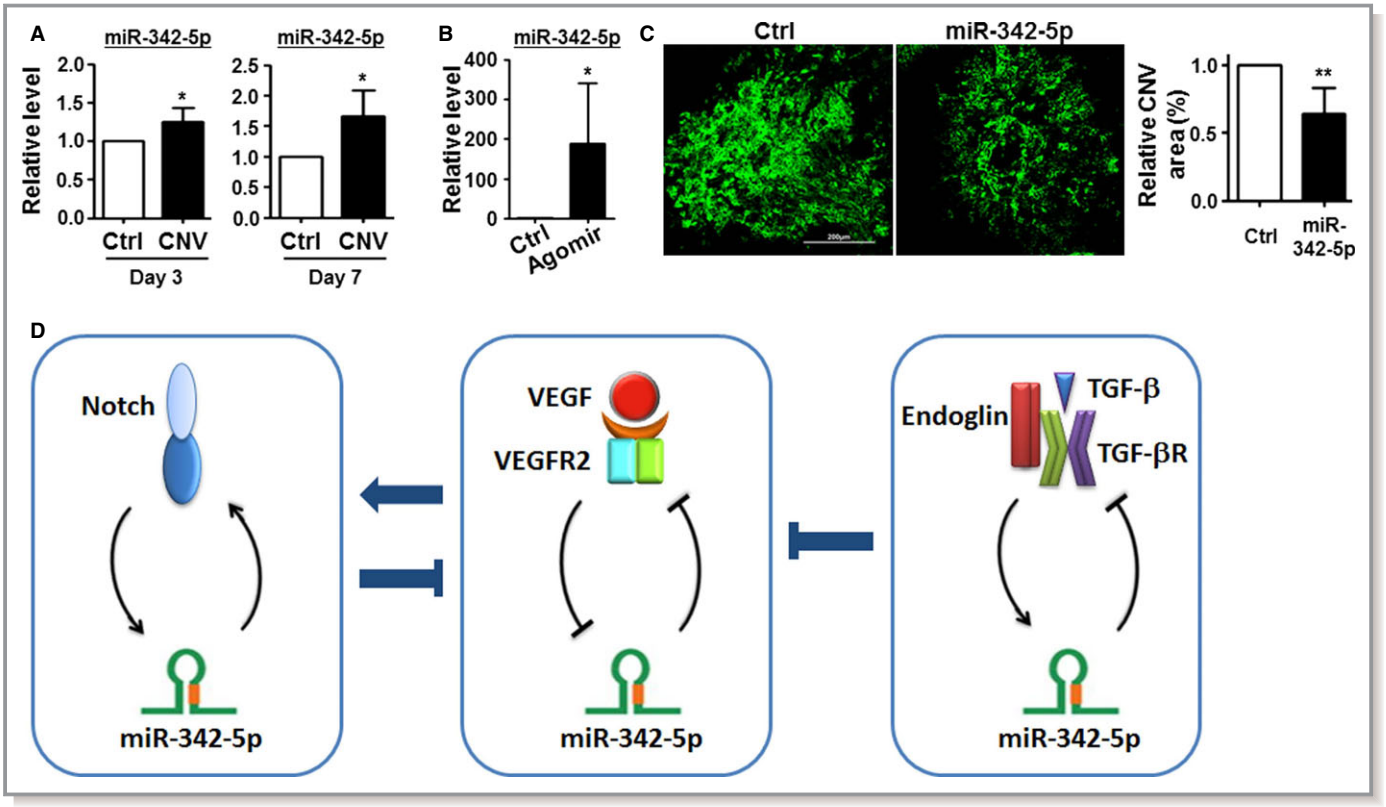

Figure 8. miR-342-5p inhibited vascularization in CNV. A, Healthy adult mice underwent laser-induced CNV. The choroid membranes of the mice were collected on days 3 and 7 after laser injury, and the expression of miR-342-5p and *EVL* mRNA was compared with the control group. B, Mice underwent laser-induced CNV and were injected intravitreally with miR-342-5p agomir. The level of miR-342-5p was determined with quantitative reverse transcription polymerase chain reaction. C, Healthy adult mice underwent laser-induced CNV. miR-342-5p agomir or control was injected intravitreally 24 hours after laser burns. Choroid membranes were collected on day 7 and stained with isolectin B4. The relative CNV areas were compared between the 2 groups. D, A model showing that miR-342-5p functions as a multieffect repressor of angiogenesis in ECs. The expression of miR-342-5p is downregulated by VEGFR2 signaling through Akt and upregulated by Notch signaling and TGF-β treatment. This miRNA represses angiogenesis by suppressing VEGFR and endoglin-mediated TGF-β receptor signaling, upregulating Dll4, and downregulating jagged 1 in Notch signaling, leading to decreased EC proliferation, migration, and probably increased endothelial–mesenchymal transition. Bars indicate mean±SD (n=5), $*P<0.05$, $**P<0.01$. CNV indicates choroidal neovascularization; Ctrl, control; EC, endothelial cell; TGF-β, transforming growth factor β; VEGF, vascular endothelial growth factor.

during angiogenesis (Figure 8D). Overexpression of miR-342-5p reduced the length of sprouts while maintaining the number of sprouts, suggesting that miR-342-5p primarily inhibited sprout growth. This finding is consistent with the decreased cell proliferation in HUVECs transfected with miR-342-5p and in angiogenic retinal vasculature of neonatal pups injected intravitreally with miR-342-5p. miR-342-5p could inhibit EC proliferation through 2 mechanisms. One is that miR-342-5p attenuated VEGF-induced Akt phosporylation, which is a major signal transduction event downstream from VEGFR2 during angiogenesis.[6,7] Moreover, Wei et al reported that miR-342-5p directly targets Akt1 through its 3′ UTR. They found that miR-342-5p in macrophages promoted atherosclerosis and enhanced the inflammatory stimulation of macrophages by suppressing the Akt1-mediated inhibition of miR-155 expression.[36]

Another mechanism underlying miR-342-5p–mediated proliferation inhibition in ECs could be the downregulation of endoglin, which we identified as a direct target of miR-342-5p in the current study. Endoglin serves as an auxiliary receptor for TGF-β signaling and is predominantly expressed in proliferating ECs and in tissues undergoing angiogenesis.[37] Endoglin promotes ALK1-mediated Smad1/5 signaling and inhibits ALK5-mediated Smad2/3 signaling, leading to enhanced EC proliferation and angiogenesis.[17,18,38,39] Inhibiting endoglin expression by specific knockdown inhibits TGF-β/ALK1 signaling and potentiates TGF-β/ALK5 signaling,[40,41] resulting in reduced proliferation.[19] Our data showed that overexpression of miR-342-5p targeting endoglin inhibited the phosphorylation of Smad1/5, whereas phosphorylation of Smad2/3 did not appear to be influenced, consistent with suppressed EC proliferation. Moreover, Lee et al reported that endoglin recruits and activates phosphoinositide 3-kinase and Akt at the cell membrane.[42] This could account for the reduced Akt phosphorylation found in ECs transfected with miR-342-5p because the level of total Akt did not appear to

change, in contrast to the situation in macrophages.[36] Further studies are needed to clarify the mechanisms of miR-342-5p–mediated reduction of Akt activation.

A consequence of miR-342-5p overexpression in ECs was increased cell migration. In the early phase of angiogenesis, a part of ECs differentiate into tip cells with filopodia. VEGF gradient-guided migration of tip cells is essential for angiogenesis.[43] Although VEGFR signaling could promote EC migration through mitogen-activated protein kinase and ERK, recent studies have highlighted the role of EndMT in tip cell differentiation and migration.[6,7,44] TGF-β signaling is the major pathway promoting EndMT.[5,11,12] Although endoglin functions as a coreceptor of TGF-β signaling, this molecule has been shown to inhibit some pathways downstream from TGF-β receptors.[20,21] Indeed, Anderberg et al recently showed that deficiency in endoglin resulted in tumor vasculature that displayed hallmarks of EndMT.[34] Our results indicated that miR-342-5p repressed the expression of endoglin through its 3′ UTR; therefore, it is likely that miR-342-5p promoted EC migration by inhibiting endoglin, leading to enhanced EndMT. Consistent with this opinion, we observed that miR-342-5p was upregulated by TGF-β treatment, and its overexpression could promote EndMT, as manifested by downregulated endothelial markers such as zonula occludens 1 and upregulated mesenchymal markers including FSP1, vimentin, Twist, β-catenin, and Snail2 (Slug). Overexpression of miR-342-5p could decrease the phosphorylation level of Smad1/5, and that could antagonize the TGF-β signal inducing EMT through Smad3 phosphorylation.[45] Interestingly, the level of CD31 was downregulated only mildly but statistically significantly by miR-342-5p overexpression in ECs, suggesting that EndMT induced by miR-342-5p could be partial, consistent with the angiogenic process.[5,44] It is an open question whether this miR-342-5p–mediated promotion of EndMT by inhibition of endoglin plays a role in physiological and pathological angiogenesis and in other related conditions such as cancer neovascularization, fibrosis, and other cardiovascular diseases.[46–48]

Although the roles and mechanisms of the major signaling pathways including VEGF, Notch, and TGF-β signals in ECs and angiogenesis have been characterized in detail, their coordinated interaction and regulation remain to be clarified.[24] VEGFR and Notch signaling pathways cooperate closely to specify and balance the differentiation of tip and stalk cells during sprouting.[8,9] Stimulating ECs with VEGFA increases the expression of Notch ligand Dll4. In reverse, Notch signaling acts as negative feedback to inhibit the expression of VEGFR2 and VEGFR3 and to increase the VEGFR1 level.[9] TGF-β also attenuates VEGFR2 transcription directly.[49] Earlier studies have also found multiple and complex interactions between Notch and TGF-β signaling.[24] Moya et al, for example, have found that Smad1/5-mediated bone morphogenetic protein signaling could cooperate with Notch signaling in the selection of tip and stalk cells during angiogenic sprouting.[50] A recent study also indicated that neuropilin 1, a coreceptor of VEGFR, suppresses the stalk cell phenotype by limiting Smad2/3 activation through ALK1 and ALK5 and that Notch signaling downregulates neuropilin 1, leading to relief of ALK1 and ALK5 inhibition to drive stalk cell behavior.[51] In this study, we found that miR-342-5p could be a central, although not strong, coordinator of Notch, VEGFR, and TGF-β signaling pathways to ultimately result in repression of VEGFR signaling (Figure 8D). This might be of significance in the decline of angiogenesis to avoid overvascularization. EndMT promoted by miR-342-5p could further facilitate vessel stabilization after angiogenic sprouting. The expression of miR-342-5p was inhibited significantly by VEGF treatment through Akt and enhanced by Notch activation and TGF-β treatment. Upregulated miR-342-5p could inhibit Akt and endoglin, which are critical signal transduction molecules of VEGFR and TGF-β signaling, respectively. This pattern of action coincided with the functional properties of miRNAs, namely, regulating by multiple signals and targeting multiple molecules. Given the sophisticated temporal and spatial regulation of cell behavior and gene expression in angiogenesis and EndMT, more studies are required to elucidate the role miR-342-5p in these processes.

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Disclosures

None.

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