MicroRNA-4530 suppresses cell proliferation and induces apoptosis by targeting RASA1 in human umbilical vein endothelial cells

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Abstract. MicroRNAs (miRNAs/miRs) are a class of endogenous and non-coding RNAs that are present in eukaryotes. In previous studies, miRNAs have been revealed to have an important role in cell growth and apoptosis. In the present study, the function of a novel and rarely studied miRNA, miR-4530, was investigated in human umbilical vein endothelial cells (HUVECs). The expression level of miR-4530 in HUVECs was investigated using reverse transcription-quantitative polymerase chain reaction following transfection with miR-4530 precursor plasmids, anti-miR-4530 plasmids and empty vector plasmids. Following this, it was revealed that overexpression of miR-4530 can suppress cell proliferation and enhance cell apoptosis. TargetScan analysis suggested that Ras p21 protein activator 1 (RASA1) is a target gene of miR-4530. The results of a dual-luciferase reporter assay also suggested that miR-4530 targets RASA1. Furthermore, the results of dual-luciferase reporter assay suggested that miR-4530 enhanced luciferase activity of the wild-type reporter, but not the mutant RASA1 reporter activity, thus suggesting that miR-4530 enhances the expression of RASA1. In addition, western blot analysis demonstrated that the protein expression level of RASA1 was enhanced following upregulation of miR-4530. The exact mechanism underlying this process has not yet been determined and requires further investigation. In addition, a RASA1 overexpression plasmid vector was transfected into HUVECs. The results suggest that overexpression of RASA1 suppresses cell growth and promotes apoptosis, which was in agreement with the results regarding the overexpression of miR-4530. To investigate how miRNA-4530 affects cellular function, numerous proteins associated with the extracellular signal-regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K)/AKT serine/threonine kinase pathways were investigated via western blot analysis. The results suggested that miRNA-4530 suppresses cell proliferation and enhances apoptosis by targeting RASA1 via the ERK/MAPK and PI3K/AKT signaling pathways.

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Introduction

MicroRNAs (miRNAs/miRs) are highly conserved, endogenous non-coding RNAs present in eukaryotes. miRNAs can degrade target mRNA or suppress the translation of target mRNA via binding to the 3' untranslated region (UTR). Depending on their targets, miRNAs can function as oncogenes or tumor suppressor genes in different cancers (1). Increasing research has suggested that aberrant expression/functions of miRNAs are associated with the progression of cancer and may serve as novel biomarkers for diagnosis and prognosis in cancers, and may be potential novel therapeutic agents for tumor suppression (2-5). miRNAs are involved in the regulation of cellular functions, including cell proliferation and apoptosis (6). For example, miRNA-101 inhibits cell proliferation and induces apoptosis, miRNA let-7c suppresses cell proliferation and induces cell cycle arrest, and miR-503 inhibits cell growth. These miRNAs all bind to the 3'UTR of target genes and subsequently affect cellular functions (7-9).

miR-4530 is located on chromosome 19. In our previous study (10), it was revealed that miR-4530 is upregulated in the serum of patients with diabetic retinopathy. Furthermore, it was demonstrated that miR-4530 enhances angiogenesis in endothelial cells and breast carcinoma cells. In addition, suppression of cell growth and promotion of apoptosis, induced by overexpression of miR-4530, was demonstrated in breast carcinoma cells (10). However, to the best of our knowledge, the effects of miR-4530 in human umbilical vein endothelial cells (HUVECs) has not yet been investigated. Furthermore, the molecular mechanism of miR-4530 associated with cell functions has not yet been determined. Therefore, the present

study aimed to investigate the role of miR-4530 in the cellular function of HUVECs.

Analysis using TargetScan has suggested that Ras p21 protein activator 1 (RASA1) is a target gene of hsa-miR-4530 in humans. The predominant function of RASA1 is to transform Ras from its active guanosine-5'-triphospate (GTP)-bound form into its inactive guanosine diphosphate-bound form by enhancement of the endogenous GTPase activity of Ras via association with its C-terminal GTPase activating protein (GAP) domain (11). In previous studies, miR-182, miR-31 and miR-223 were demonstrated to regulate cell proliferation, apoptosis, migration and angiogenesis via association with RASA1 (12-14). Thus, as an important Ras GAP, a mutation within the RASA1 gene may result in the progression of numerous diseases. Furthermore, the enhancement of cell proliferation and tube formation in endothelial cells resulting from RASA1 depletion has been previously reported (15). The present study aimed to investigate whether miR-4530 can also regulate cell growth and apoptosis by targeting RASA1 in endothelial cells.

Materials and methods

Endothelial cell culture. The two cell lines used in the present study were 293T cells and HUVECs, purchased from the Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences (Shanghai, China). Total 293T cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) containing 10% fetal bovine serum (FBS; Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) and 1% penicillin/streptomycin. RPMI-1640 medium (Invitrogen; Thermo Fisher Scientific, Inc.), containing 10% FBS and 1% penicillin/streptomycin, was used for culturing HUVECs. All cells were placed in a humidified incubator at 37˚C containing 5% CO_2.

Construction of plasmids and transfection into HUVEC cells. The pPG/miR/enhanced green fluorescent protein (EGFP), pPG-miR4530-EGFP and pPG-miR4530sponge-EGFP plasmids were purchased from Chang Jing Bio-Tech, Ltd. (Changsha, China). The sequences inserted into the plasmids were as follows: miR-4530, 5'-AATTCGCCCAGCAGGACG GGAGCGGTTTTGGCCACTGACTGACCGCTCCCGCTG CTGGGCA-3'; anti-miR-4530, 5'-AATTCCGCTCCCGTC CTGCTGGGCGATCGCTCCCGTCCTGCTGGGACCGGT CGCTCCCGTCCTGCTGGGTCACCGCTCCCGTCCTGC TGGGTTTTTTACGG-3'. Furthermore, the vector upregulating RASA1 (GV230, *Xho*I/*Kpn*I) and the negative control (a random sequence) were purchased from Shanghai GeneChem Co., Ltd. (Shanghai, China). The full-length open reading frame of RASA1 (GeneBank accession no. NM_002890) was amplified using the following primers: RASA1, forward, 5'-TACCGGACTCAGATCTCGAGCGCC ACCATCGATGGCGGCCGAGGCCGGCAGTG-3' and reverse, 5'-GATCCCGGGCCCGCGGTACCGTCCTGA CATCATTGGTTTTTGTATACTGG-3'. The polymerase chain reaction (PCR) product was then inserted into the expression vector GV230. In accordance with the manufacturer's instructions, transfection was performed using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.). Briefly, cells were added to a 6-well plate, cultured overnight until a ~70% convergence degree was reached, and then resuspended in serum-free RPMI-1640 (Invitrogen; Thermo Fisher Scientific, Inc.). Lipofectamine® 3000 was diluted in serum-free RIPM-1640 and mixed with the diluted miRNA or plasmid and P3000 (Invitrogen; Thermo Fisher Scientific, Inc.), incubated at room temperature for 5 min, and then added to the cellular suspension. Following 4-6 h of incubation, the medium was replaced with fresh RPMI-1640 containing 10% FBS (Sigma-Aldrich; Merck KGaA). According to the resistance of the different vectors, blasticidin (for pPG-miR4530-EGFP and pPG-miR4530sponge-EGFP plasmids; Sigma-Aldrich; Merck KGaA; 15 μg/ml) and G418 (for GV230; Gibco; Thermo Fisher Scientific, Inc.; 20 μg/ml) were also used to screen for stable cell lines.

Reverse transcription-quantitative PCR (RT-qPCR). TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.) was used to extract total RNA from HUVECs according to the manufacturer's instructions. Following this, 1,000 or 2,000 μg total RNA was reverse transcribed into miRNA-cDNA using All-in-One miRNA First-Strand cDNA Synthesiskit (GeneCopoeia, Inc., Rockville, MD, USA) according to the manufacturer's protocol. A total of 1,000 μg RNA was reverse-transcribed into mRNA-cDNA using PrimeScriptRT Reagent kit with gDNA Eraser (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's protocol. In addition, qPCR was performed using the SYBR-Green PCR kit (Takara Biotechnology Co., Ltd.) on the StepOne-Plus Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) to detect miR-4530 and RASA1 mRNA expression. The following primers were used: miR-4530 forward, 5'-TGCTGTCAACGATACGCTACG-3' and reverse, 5'-TGC TGTCAACGATACGCTACG-3'; RASA1 forward, 5'-ACT TGACAGAACGATAGCAGAAG-3' and reverse, 5'-GCCTCC GATCACTCTCTCTTA-3'. Human U6 RNA was used as an internal control for the normalization of miRNA expression. The internal control used for the normalization of mRNA was GAPDH. The primers used for GAPDH amplification were: GAPDH sense, 5'-GGAGTCCACTGGCGTCTT-3' and antisense, 5'-ATCTTGAGGCTGTTGTCATAC-3'. The thermocycling conditions were: 95˚C for 10 min, 95˚C for 10 sec and 55˚C for 10 sec, with a final step of 72˚C for 30 sec. Then steps 2-4 were repeated for 40 cycles followed by a melt curve program for 60 min. The expression levels were quantified using the $2^{-\Delta\Delta Cq}$ method (16). Primers of U6 were purchased from GeneCopoeia, Inc. (cat. no. HmiRQP9001; 2 μM).

Dual-luciferase reporter assay. TargetScan was used to predict the potential targets of miR-4530 (9,17-19). In TargetScanRelease 6.2 database, the human species was selected and the miRNA name was entered as 'has-miR-4530'. The potential targets were selected. The analysis predicted that RASA1 is a direct target gene of miR-4530. In order to verify the results of this analysis, dual-luciferase reporter assays were performed. Human RASA1 mRNA 3'UTR WT and mutated miR-4530 human RASA1 mRNA 3'UTR sequences were amplified and then inserted into pmiR-reporter plasmids. The following pmirGLO Dual-Luciferase miRNA target expression vectors were purchased from Chang Jing Bio-Tech, Ltd. (Changsha, China): PmirGLO-3'

UTR-WT, 5'-TGATGTGTGAGCTATGCAAACAAAATCCAAGATTCTGCTGGTGAATAACTATGC-3' and pmirGLO-3'UTR-MUT, 5'-TGATGTGTGAGCTATGCAAACAAAATCCAAGATTCTTACGGTGAATAACTATGC-3'. miRNA-4530 mimics (oligonucleotide sequence, 5'-CCCAGCAGGACGGGAGCG-3') and NC mimics (random sequence, 5'-CAGUACUUUUGUGUAGUACAA-3') were also purchased from Chang Jing Bio-Tech, Ltd. (Changsha, China). miR-4530 mimics simulated the endogenous and mature body of the miR-4530 sequence, and enhanced its expression.

The following three vectors were co-transfected into ~1x10^5 293T cells in 24-well plates using Lipofectamine® 3000 (Invitrogen; Thermo Fisher Scientific, Inc.): miR-4530 mimics + pmir-GLO-3'UTR wild-type (Shanghai GenePharma Co., Ltd; 25 ng), miR-4530 mimics + pmir-GLO-3'UTR-mutant (Shanghai GenePharma Co., Ltd; 25 ng) and negative control (NC)-mimics + pmir-GLO-WT (25 ng). At 24 h post-transfection, cells were lysed using a passive lysis buffer in a dual-luciferase reporter assay kit (Promega Corporation, Madison, WI, USA). Luciferase signals were subsequently determined using a Tecan M1000 microplate reader (Thermo Fisher Scientific, Inc.). The internal control used for normalization was comparison with *Renilla* luciferase activity, and the strength of firefly luciferase activity represented the expression of firefly luciferase.

Colony formation assay. Then 3 groups [pPG/miR/enhanced green fluorescent protein (EGFP), pPG-miR4530-EGFP and pPG-miR4530sponge-EGFP] of cells were digested using pancreatin enzymes, and then 500 cells were counted from each group and seeded into 6-well plates. Medium was replaced with fresh RPMI-1640 containing 10% FBS every 2 days. Following 14-16 days of incubation, cells were washed twice with PBS and then fixed with 4% paraformaldehyde for 15 min at room temperature. Following this, cells were stained with 0.1% crystal violet (Beyotime Institute of Biotechnology, Haimen, China) for 15 min and washed using high pressure water. The colony formation assay was performed in triplicate and the results were imaged using a digital camera.

Cell proliferation assay. Cell growth was determined using Cell Counting Kit-8 (CCK8; Dojindo Molecular Technologies, Inc., Kumamoto, Japan) assays. Stable transfected cells were seeded into 96-well plates (2,000 cells/well) and maintained at 37˚C; the medium was replaced with fresh RPMI-1640 every 2 days. Then, 3 wells were used for each group and PBS was added to all other empty wells in order to decrease error. At 24, 48, 72, 96 and 120 h time intervals, the medium was replaced with 100 μl fresh serum-free RPMI-1640, 10 μl CCK8 solution was added to each well, and plates were then incubated at 37˚C for 1 h. Following this, all plates were analyzed at wavelength of 450 nm using a microplate reader (Thermo Fisher Scientific, Inc.). To confirm that the miR-4530 promotes cell apoptosis, PI3K/AKT inhibitor (LY294002; Cell Signaling Technology, Danvers, MA, USA) was added to the stable cell lines and the cell proliferation investigated by CCK8. First, stable transfected cells were seeded into 96-well plates. After 24 h, the inhibitor was diluted in concentrations of 5, 10, 20 and 40 μM using 1640 medium. Then 100 μl was added to the cells. The specific steps of CCK8 are the same as described above. To confirm that upregulation of miR-4530 inhibited cell growth, a response experiment was required. Stable transfected cells were seeded into 6-well plates and after 24 h, ERK/MAPK inhibitor (U126; Merck KGaA) was diluted to the concentration of 5, 10, 20 or 40 μM using 1640 medium and 2 ml added to the plates. Cell apoptosis was detected as below. Each assay was performed in triplicate.

Cell cycle and cell apoptosis analysis. Stably transfected cells were collected by pancreatin enzymes and centrifuged at 1,200 x g for 5 min at room temperature. Cells were washed twice in the process of cell collection. Cells were fixed in 70% ethanol at 4˚C overnight; that cells did not cluster was crucial to the experiment. Cells were washed twice using PBS, and the cells were then re-suspended in 160 μl 0.5 mg/ml RNase A (Nanjing KeyGen Biotech Co., Ltd., Nanjing, China) and incubated at 37˚C for 30 min. Following this, cells were stained using 50 μmol propidium iodide (Nanjing KeyGen Biotech Co., Ltd.) and then analyzed via flow cytometry using a flow cytometer (BD Biosciences, Franklin Lakes, NJ, USA). The Annexin V-allophycocyanin (APC)/Propidium Iodide kit (Nanjing KeyGen Biotech Co., Ltd.) was used to analyze cell apoptosis. Cells were collected using pancreatin enzymes, washed using PBS and stained using the Annexin V-APC/Propidium Iodide kit (Nanjing KeyGen Biotech Co., Ltd.) according to the manufacturer's protocols. The cells were analyzed using flow cytometry (BD Biosciences). Data on cell apoptosis were analyzed using FlowJo version 7.6 (FlowJo LLC, Ashland, OR, USA) and the data of cell cycle was analyzed by ModFit LT 3.1 (Verity Software House, Inc., Topsham, ME, USA).

Western blotting. Total protein was extracted using radioimmunoprecipitation buffer (P0013D; Beyotime Institute of Biotechnology, Haimen, China) containing 1% phenylmethane sulfonyl fluoride. Lysates were transferred to clean Eppendorf tubes and then centrifuged at 12,000 x g for 10 min at 4˚C to remove cellular debris. The total protein concentration was determined using the Bradford assay (Beyotime Institute of Biotechnology) in accordance with the manufacturer's instructions. Electrophoresis was used to separate 30 μg protein samples using a 10% SDS-PAGE gel. Proteins were then transferred to polyvinylidene fluoride membranes (Merck KGaA). Proteins were then blocked with milk (Bio-Rad Laboratories, Inc., Hercules, CA, USA) for 2-3 h at room temperature to prevent non-specific binding. Membranes were then incubated overnight at 4˚C with primary antibodies against RASA1 (1:2,000; cat. no. ab40677; Abcam, Cambridge, UK), phospho-Ser473 AKT serine threonine kinase (p-AKT; 1:2,000; cat. no. 4060; Cell Signaling Technology, Inc.), p-Ser308 AKT (1:1,000; cat. no. 2965; Cell Signaling Technology, Inc.), B-cell lymphoma-2 (BCL-2; 1:2,000; cat. no. ab182858; Abcam), BCL-2-like protein 4 (BAX; 1:1,000; cat. no. ab32503; Abcam), AKT (1:1,000; cat. no. 9272; Cell Signaling Technology, Inc.), GAPDH (1:6,000; cat. no. A0208; Beyotime Institute of Biotechnology) and phosphorylated-extracellular signal-regulated kinase (p-ERK; 1:2,000; cat. no. 9910; Cell Signaling Technology, Inc.) Following this, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000 in TBST; cat. no. A0208; Beyotime Institute of

Biotechnology) for 1 h at room temperature, and then washed with Tris-buffered saline with 0.05% (v/v) Tween-20 for 1 h. Band signals were visualized using an enhanced chemiluminescent kit (Beyotime Institute of Biotechnology) according to the manufacturer's protocol. ImageJ version 1.44 software (National Institutes of Health, Bethesda, MD, USA) was used to analyze the gray level of every band.

Statistical analysis. Comparative analysis between groups was determined by use of either the two-tailed Student's t-test or one way analysis of variance followed by the Bonferroni test. $P<0.05$ was considered to indicate a statistically significant difference. All experiments were conducted in triplicate, and data are expressed as mean ± standard deviation.

Results

Overexpression of miR-4530 results in suppression of cell proliferation and enhancement of apoptosis, resulting in G2/M arrest in HUVECs. To investigate the role of miR-4530 in HUVECs, the cell proliferation of stable transfected HUVECs was investigated using CCK8 assays and colony formation assays. The results of the CCK8 assays suggested that the proliferation rate of HUVECs was significantly suppressed following overexpression of miR-4530 (Fig. 1A). Furthermore, colony formation was significantly suppressed following overexpression of miR-4530, and markedly enhanced following knockdown of miR-4530 (Fig. 1B). In order to investigate the effects of miR-4530 on HUVECs, the cell cycle progression of miR-4530 transfected cells was investigated. The results suggested that overexpression of miR-4530 results in G2/M cell cycle arrest. Furthermore, the results demonstrated that 28.26% of cells in the overexpression miR-4530 group were in the G2/M phase, 14.80% cells in the control group were in the G2/M phase and 9.64% cells of the miR-4530 knockdown group were in the G2/M phase (Fig. 1C). Previous studies have suggested that cells arrest in the G2/M phase and then undergo apoptosis following treatment with miR-4530 (20). Thus, the results of the present study suggest that overexpression of miR-4530 suppresses the proliferation of HUVECs by blocking the G2/M stage transition. Furthermore, cell apoptosis was also investigated using flow cytometry. Stable transfected HUVECs were stained using the Annexin V-APC/Propidium Iodide Kit. The results suggested that miR-4530 significantly enhances apoptosis in HUVECs cells compared with the negative control (Fig. 1D). However, anti-miR-4530 did not affect apoptosis in HUVEC and it was hypothesized that the expression of miR-4530 was low in HUVEC. When the expression of miR-4530 was decreased its effects on downstream proteins were possibly less obvious and this is confirmed by the results of subsequent experiments. In general, these results suggest that miR-4530 is involved in apoptotic pathways.

RASA1 is a direct target gene of miR-4530 in HUVECs. miRNA database (TargetScan) were analyzed to identify potential targets and binding sites of miR-4530. Analysis using TargetScan suggested that RASA1 is a target of miR-4530, with a high degree of complementarity. The potential binding site is located at bases 148-152 within the RASA1 3'UTR (Fig. 2A). A dual-luciferase reporter assay was performed to further investigate the association between miR-4530 and RASA1 in 293T cells. The sequences of Firefly luciferase were followed by either the 3'UTR-WT or 3'UTR-MUT of RASA1 in pmirGLO-3'UTR-WT or pmirGLO-3'UTR-MUT. *Renilla* luciferase signal was used as an internal control, and the activity of Firefly luciferase decreased following binding of miR-4530 to the RASA1 3'UTR. The results of the dual-luciferase reporter assay demonstrated that the relative luciferase activity of the WT group was significantly enhanced compared with the other two groups, thus suggesting that miR-4530 targets the 3'UTR of RASA1 and promotes its expression. However, following mutation of the binding locus, there was no significant change exhibited in the RASA1-MUT compared with the control. The mutation of three bases within the RASA1 3'UTR was considered to sufficiently inhibit binding with miR-4530, as there was no significant difference between the RASA1-MUT group and the control group regarding relative luciferase activity, and miRNAs regulate their target gene by binding to few genes of 3'UTR. Therefore, the results suggested that residual binding does not occur between miR-4530 and RASA1-MUT. In conclusion, the results suggest that miR-4530 enhances the Firefly luciferase signal, and this effect is reversible following mutation of the miR-4530 binding site in the RASA1 3'UTR (Fig. 2B). To further investigate the increase of RASA1 expression in HUVECs following treatment with miR-4530, stable transfected HUVEC cell lines were constructed. Each cell line contained a stable transfected miR-4530 precursor group, an anti-miR-4530 group and a negative control group. Each group was confirmed by determining the expression levels of miR-4530. It was demonstrated that the expression level of miR-4530 was enhanced following transfection with the miR-4530 precursor group compared with the negative control group. In addition, the expression level of miR-4530 in the anti-miR-4530 group was suppressed compared with the negative control group (Fig. 2C). Furthermore, RT-qPCR and western blot analysis suggested that RASA1 mRNA and protein expression were significantly increased following miR-4530 overexpression in HUVECs (Fig. 2D and E). TargetScan analysis suggested that miR-4530 has numerous target genes. Whether miR-4530 targets specific genes that negatively regulate RASA1, or whether miR-4530 regulates the expression of RASA1 via alternative mechanisms that promote the expression of RASA1, has not yet been determined.

miR-4530-regulated RASA1 is involved in the proliferation and apoptosis of HUVECs. In previous studies, numerous miRNAs have been demonstrated to regulate cell proliferation, apoptosis, migration and angiogenesis via association with RASA1 (12-14). To investigate whether miR-4530-regulated RASA1 is involved in cell proliferation and apoptosis, a RASA1 overexpression plasmid and a negative control plasmid were transfected into HUVECs. The expression of RASA1 mRNA and protein were significantly enhanced in HUVECs transfected with the RASA1 overexpression plasmid (Fig. 3A and B). Furthermore, CCK8 assays were performed to investigate cell growth. The results demonstrated that cell proliferation was significantly suppressed following overexpression of RASA1 compared with the negative control, which is consistent with results of the miR-4530 overexpression analyses (Fig. 3C). In addition, the results of the Annexin V-FITC assay revealed that apoptosis was significantly enhanced in HUVECs following

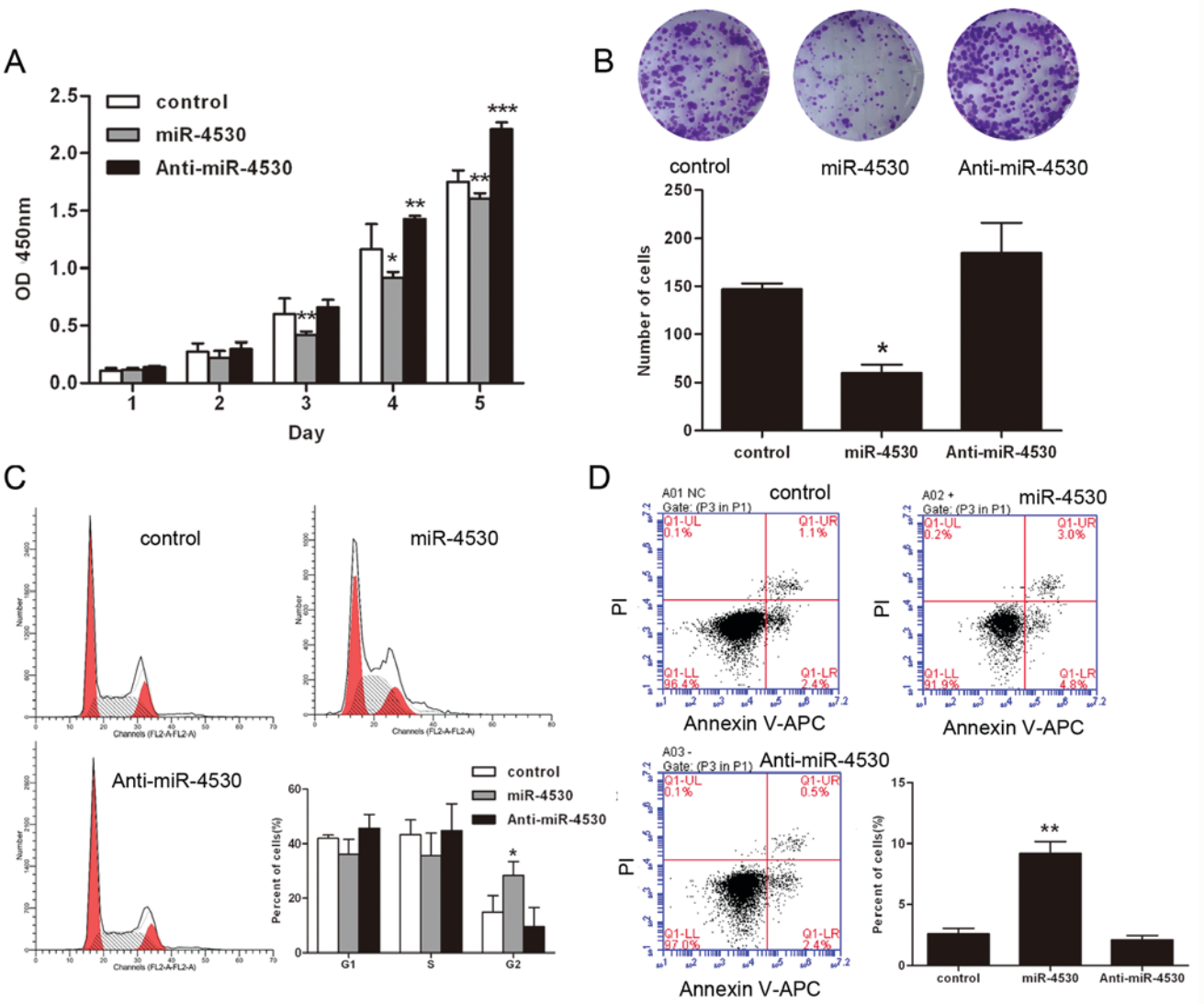

Figure 1. Suppression of cell proliferation and enhancement of apoptosis are caused by miR-4530 overexpression in HUVECs. (A) Cell proliferation was determined by the Cell Counting Kit-8 assay. (B) Colony formation was significantly suppressed following overexpression of miR-4530, and enhanced following knockdown of miR-4530. (C) Overexpression of miR-4530 in HUVECs induces G2/M cell cycle arrest determined by flow cytometry. (D) Cell apoptosis was analyzed via flow cytometry, and it was revealed that overexpression of miR-4530 enhances apoptosis in HUVECs. *P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control. HUVEC, human umbilical vein endothelial cell; miR, microRNA; OD, optical density; PI, propidium iodide; APC, allophycocyanin.

upregulation of RASA1 (Fig. 3D). Therefore, the results suggest that suppression of cell proliferation and enhancement of apoptosis following miR-4530 overexpression are dependent upon the association between miR-4530 and RASA1.

Phosphoinositide 3-kinase (PI3K)/AKT signal transduction pathway is involved in cell apoptosis induced by miR-4530 overexpression. To elucidate the signaling pathways involved in miR-4530-associated regulation of cell growth in HUVECs, the PI3K/AKT pathway, an anti-apoptosis signaling pathway, was investigated. The level of p-Ser473 AKT and p-Thr308 AKT were suppressed in the miR-4530 overexpression group compared with the negative control. However, the levels of p-Ser473 AKT and p-Thr308 AKT in HUVECs did not exhibit a marked change following transfection with the miR-4530 inhibitor compared with the negative control (Fig. 4A). In addition, the expression of BAX was significantly enhanced and the expression of BCL-2 was significantly suppressed in HUVECs overexpressing miR-4530 compared with the negative control (Fig. 4B). p-Ser473 AKT, p-Thr308 AKT, BAX and BCL-2 are all downstream effectors of the PI3K/AKT signaling pathway associated with cell apoptosis. These results suggest that the PI3K/AKT signal transduction pathway is involved in cell apoptosis resulting from miR-4530 overexpression; however, there were no marked differences in the expression of proteins associated with the PI3K/AKT pathway following the downregulation of miR-4530. In order to further investigate the conclusion, stable transfected cells were treated with a PI3K/AKT inhibitor at different concentrations. Following treatment with LY294002, the percentage of apoptotic cells increased in a dose-dependent manner, and the difference between the miR-4530 overexpression group and control group was decreased in a dose-dependent manner (Fig. 4C). In conclusion, the results suggest that miR-4530 enhances cell apoptosis via the PI3K/AKT pathway.

Suppression of cell proliferation via miR-4530 overexpression is associated with the ERK/mitogen-activated protein kinase

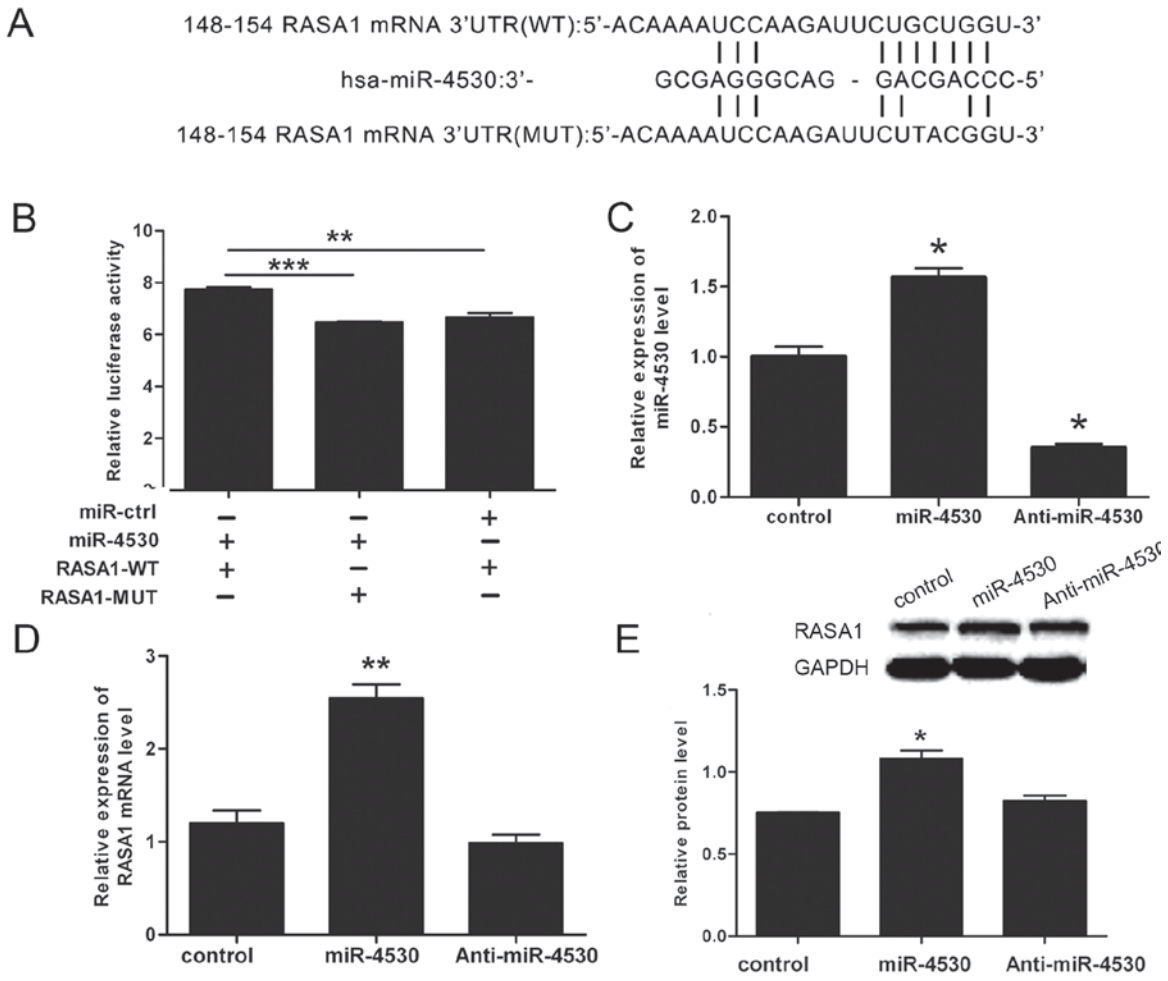

Figure 2. miR-4530 upregulates RASA1 expression in HUVECs. (A) Target sequence of miR-4530 in WT RASA1 3'UTR, and the sequence of MUT 3'UTR of RASA1. (B) miR-4530 mimics enhanced wild-type, but not mutant, RASA1 reporter activity in 293T cells. (C) The expression of miR-4530 was increased by transfection with miR-4530 precursor group compared with the negative control, and transfection with anti-miR-4530 did not exhibit a significant difference in the relative expression of miR-4530 compared with the negative control. (D) Expression of RASA1 mRNA was increased following overexpression of miR-4530. (E) miR-4530 upregulates endogenous RASA1 protein expression HUVECs, and densitometric analysis revealed that the difference in RASA1 expression between the miR-4530 overexpression group and the negative control group was statistically significant. *P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control. HUVEC, human umbilical vein endothelial cell; RASA1, Ras p21 protein activator 1; 3'UTR, 3' untranslated region; WT, wild-type; MUT, mutant; miR, microRNA.

(MAPK) pathway. To determine the pathway associated with the suppression of cell proliferation by miR-4530 in HUVECs, the ERK/MAPK pathway was investigated. The ERK/MAPK signaling pathway regulates numerous important cellular progresses (21). Upregulation of the ERK/MAPK pathway has previously been revealed to enhance cell proliferation (22). In the present study, the level of p-ERK was suppressed following overexpression of miR-4530 compared with the negative control, however, the expression of RAS did not exhibit a marked difference following miR-4530 overexpression (Fig. 5A). Densitometric analysis of western blot analyses demonstrated that the difference in the level of p-ERK between the miR-4530 overexpression group compared with the negative control was statistically significant (Fig. 5B). Furthermore, an ERK/MAPK inhibitor (U126) was added to stable transfected cells. The growth of HUVECs was revealed to be suppressed following treatment with U126 in a dose-dependent manner, and the difference between the miR-4530 overexpression group and the control group decreased in a dose-dependent manner (Fig. 5C). Thus, the results suggest that suppression of HUVEC cell proliferation following miR-4530 overexpression is associated with the ERK pathway, however, there were no marked differences associated with the MAPK/ERK pathway following the downregulation of miR-4530.

Discussion

In our previous study, it was demonstrated that miR-4530 is upregulated in the serum of patients with diabetic retinopathy (10). Diabetic retinopathy is a prevalent eye complication resulting from diabetes. According to a number of previous studies, it has been demonstrated that the occurrence and progression of diabetic retinopathy is associated with angiogenesis and apoptosis in numerous cell types, including capillary endothelial cells, peripheral cells and nerve cells (23-26). It has previously been reported that overexpression of miR-4530 enhances angiogenesis in endothelial cells (27) and that the enhanced proliferation and suppression of apoptosis exacerbates angiogenesis (28). However, the results of the present study revealed that overexpression of miR-4530 enhances

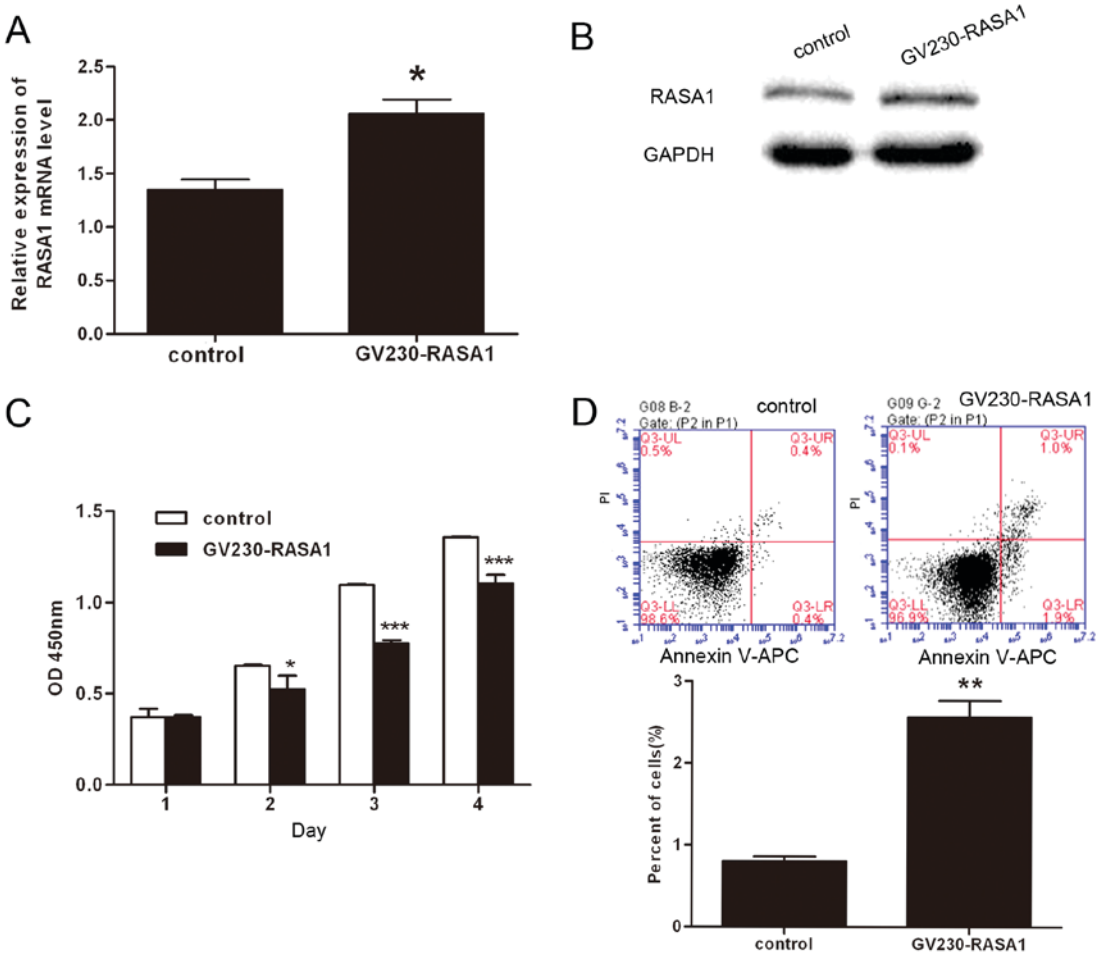

Figure 3. Upregulation of RASA1 suppresses cell growth and enhances apoptosis. (A) Expression of RASA1 mRNA was enhanced following overexpression of RASA1. (B) Expression of RASA1 was increased following upregulation of RASA1 compared with the negative control. (C) Cell proliferation was investigated by performing Cell Counting Kit-8 assays, and it was demonstrated that overexpression of RASA1 significantly suppresses cell growth. (D) Cell apoptosis was analyzed by flow cytometry, and overexpression of RASA1 was revealed to significantly enhance apoptosis in human umbilical vein endothelial cells. *P<0.05; **P<0.01; ***P<0.001. RASA1, Ras p21 protein activator 1; OD, optical density; APC, allophycocyanin.

apoptosis and suppresses cell growth in HUVECs. According to the results of the present study, miRNA-4530 may have an important role in the pathogenesis of diabetic retinopathy due to enhancement of apoptosis. Further investigation into the mechanisms underlying the association between miR-4530 and the suppression and enhancement of cell proliferation and apoptosis, respectively, may identify novel therapeutic approaches for the treatment of diabetic retinopathy.

TargetScan analysis and the results of the dual-luciferase reporter assay revealed that RASA1 is a direct target gene of miR-4530 in HUVECs. RASA1 regulates numerous cell functions, including migration, growth, apoptosis and angiogenesis, via the Ras signaling pathway (29-31). The results of the present study revealed that RASA1 has an important role in the regulation of cell proliferation and apoptosis. RT-qPCR was used to determine the expression level of miR-4530 and RASA1 mRNA following transfection with pmiR-reporter plasmids. It has previously been demonstrated that miRNAs can suppress the expression of target genes by targeting associated 3'UTR sequences (32). However, in the present study, the expression levels of RASA1 mRNA and protein were enhanced following overexpression of miR-4530. An miRNA may have numerous target genes, and a target gene can be regulated by numerous miRNAs. miR-4530 regulates a number of proteins upstream of RASA1, which subsequently negatively regulate the expression of RASA1. Thus, miR-4530 enhances the expression of RASA1, however, the exact molecular mechanism underlying this process remains unclear. Previous studies have demonstrated that overexpression of miRNA enhances the expression of target proteins in smooth muscle cells (33). A further study revealed that miRNAs can transform their inhibitory roles to enhancer roles; for example, miR-369-3 enhances the translation of its target gene. miRNAs that contain AU rich elements and conserved sequence targets predominantly upregulate the translation of target proteins, particularly in a period of cell cycle arrest following cell processing (32). Following overexpression of miR-4530, G2/M arrest was determined by flow cytometry analysis. To determine the mechanism underlying the regulatory functions of miR-4530, ERK/MAPK and PI3K/AKT signaling pathways were investigated.

The activation of the PI3K/AKT pathway can suppress cell apoptosis via the inactivation of BAX (34) and caspase-9 (35). In the present study, the levels of p-S473 AKT and p-T308 AKT were suppressed following miR-4530 overexpression, and the expression of total AKT did not exhibit a significant difference following miR-4530 overexpression. Furthermore,

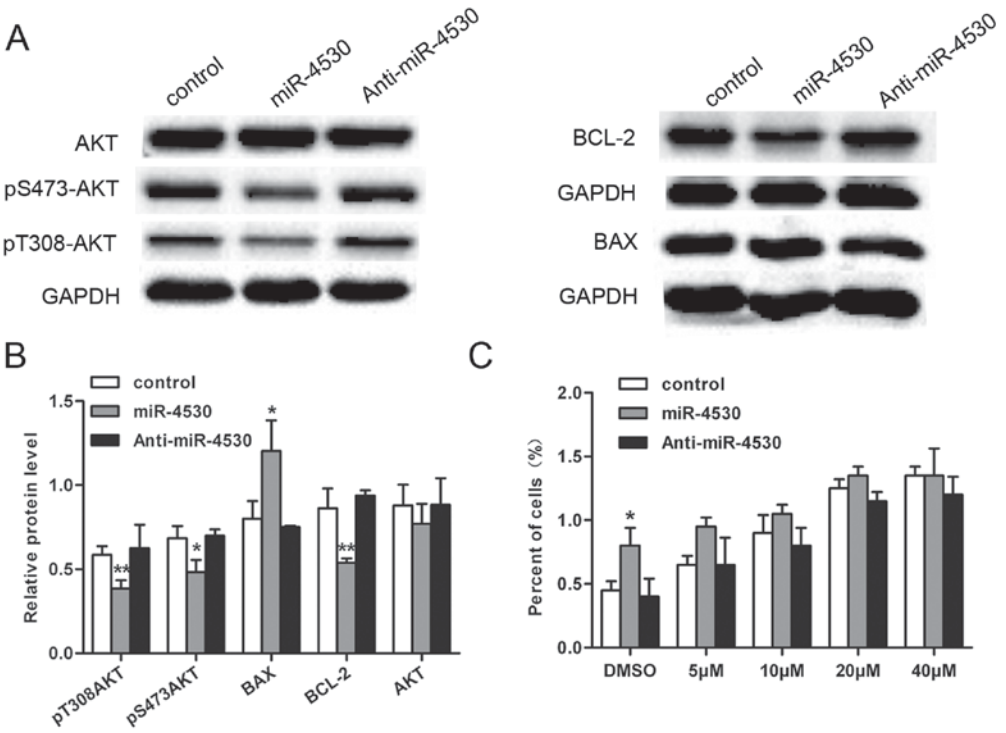

Figure 4. Expression levels of AKT, p-Thr308 AKT, p-Ser473 AKT, BAX and BCL-2 were investigated. (A) The expression levels of p-Thr308 AKT, p-Ser473 AKT, BCL-2 and AKT were suppressed following miR-4530 overexpression compared with the negative control; whereas expression of BAX was significantly enhanced following miR-4530 overexpression compared with the negative control. (B) Densitometric analysis of p-Thr308 AKT, p-S473AKT, BAX and BCL-2. (C) Following addition of the PI3K/AKT inhibitor, apoptosis was enhanced in a dose-dependent manner, and the difference between the miR-4530 overexpression and control groups was reduced in a dose-dependent manner. *P<0.05 vs. control; **P<0.01 vs. control. miR, microRNA; AKT, AKT serine/threonine kinase; p, phospho; BCL-2, B-cell lymphoma-2; BAX, BCL-2-like protein 4.

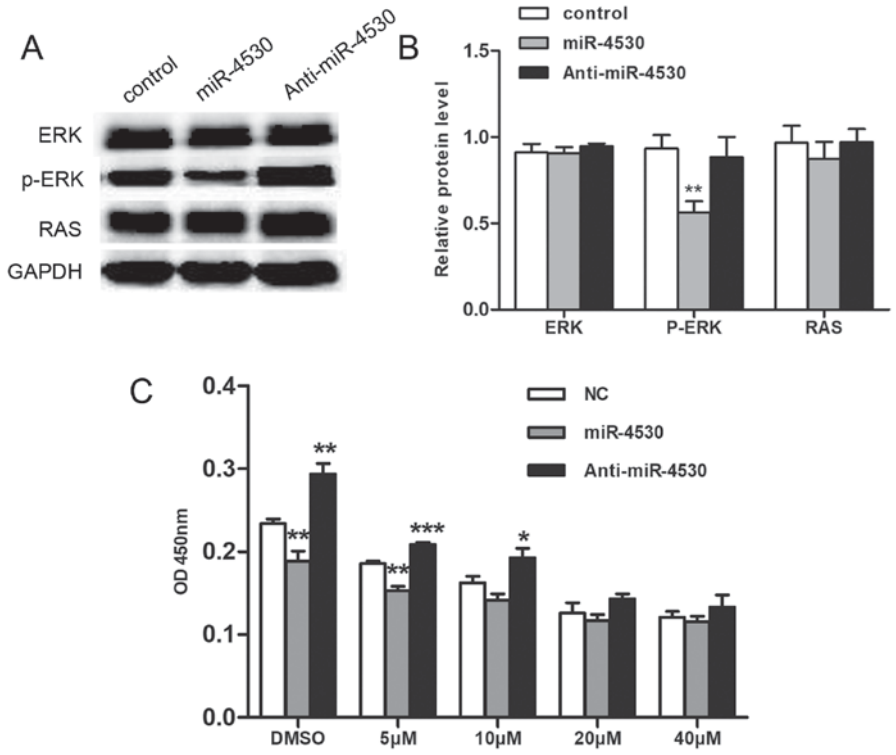

Figure 5. Protein level of p-ERK, total ERK and Ras. (A) The level of p-ERK was suppressed following overexpression of miR-4530 compared with the negative control. (B) Densitometric analysis of ERK, p-ERK and Ras revealed that the difference between the level of p-ERK in the miR-4530 overexpression group compared with the negative control group is statistically significant. (C) Following addition of the ERK inhibitor, the growth of human umbilical vein endothelial cells was suppressed in a dose-dependent manner, and the difference between the miR-4530 overexpression and control groups was reduced in a dose-dependent manner. *P<0.05 vs. control; **P<0.01 vs. control; ***P<0.001 vs. control. miR, microRNA; p-, phospho-; ERK, extracellular signal-regulated kinase; NC, negative control; OD, optical density.

it was revealed that the level of associated proteins following downregulation of miR-4530 did not exhibit a significant change compared with the negative control. The concentration of exogenous miR-4530 has been demonstrated to be low, however, other types of cells *in vivo* can also secrete miR-4530, which subsequently acts on endothelial cells (10). Furthermore, it has previously been demonstrated that cells can secrete miRNAs, which then target recipient cells, and that exogenous miRNAs can subsequently regulate target gene expression and recipient cell function (36). BAX and BCL-2 are downstream effectors of the PI3K/AKT pathway (34). In the present study, following the upregulation of miR-4530 expression in HUVECs, the results demonstrated that the expression of BAX was enhanced and the expression of BCL-2 was suppressed. Therefore, it can be suggested that the PI3K/AKT pathway is inactivated following upregulation of miR-4530, thus resulting in an increase of apoptosis compared with the negative control. Following the addition of a PI3K/AKT inhibitor into stable transfected cells, flow cytometry analysis revealed that the percentage of apoptotic cells increased in a dose-dependent manner, and the difference between the percentage of apoptotic cells of the miR-4530 overexpression and control groups reduced in a dose-dependent manner. Thus, PI3K/AKT is an important pathway associated with miR-4530 regulation of cell apoptosis. Furthermore, it can be suggested that miR-4530 targets RASA1, RASA1 subsequently acts on the Ras pathway, and then the Ras pathway targets the PI3K/AKT pathway in order to regulate cell apoptosis. Considering this, our future studies will further investigate RASA1 and its association with miR-4530 and the PI3K/AKT pathway. Such future studies will consist of a RASA1 knockdown assay, and an investigation into the effects the downregulation of RASA1 on cell proliferation and protein expression associated with the PI3K/AKT pathway.

The ERK/MAPK pathway primarily functions as a linear pathway responsible for the transmission of signals from cell surface receptors to ERK/MAPK, which then transduce signals to downstream effectors (37). The ERK/MAPK pathway has been evolutionary conserved and regulates numerous important cellular progresses, including cell growth, survival, differentiation and motility (37). Furthermore, previous studies have demonstrated that numerous proteins, including insulin-like growth factor I and metastasis associated lung adenocarcinoma transcript 1, regulate cell proliferation via the ERK/MAPK signaling pathway (38-40). Considering the association between the ERK/MAPK pathway and cell proliferation, the key proteins of the ERK/MAPK pathway were investigated in the present study. The level of p-ERK1/2 was revealed to be suppressed following miR-4530 overexpression miR-4530 compared with the negative control, and thus the results suggest that the activity of the ERK/MAPK pathway is suppressed following miR-4530 upregulation. Furthermore, an ERK inhibitor was added to the stable transfected cells. The results suggested that the growth of HUVECs was suppressed in a dose-dependent manner, and that the difference between the growth of HUVECs in the miR-4530 overexpression and control groups decreased in a dose-dependent manner following treatment with the inhibitor. In conclusion, miR-4530 was revealed to regulate cell growth via regulation of the ERK/MAPK pathway. The results indicated that increased RASA1 inactivated Ras, which inhibited ERK/MAPK signaling, resulting in reduced cell proliferation.

Studies have demonstrated that miR-4530 has an important role as a molecular marker for the diagnosis of numerous diseases. One study revealed that the expression of miR-4530 was suppressed in patients with pancreatic and biliary-tract cancers compared with healthy subjects, and that miR-4530 represents one of eight significant diagnostic markers able to identify patients with pancreatic and biliary-tract cancers (41). In addition to this, a further study demonstrated that the expression of miR-4530 was upregulated in the serum of patients with type 2 diabetes compared with healthy subjects, and that the differential expression of miR-4530 may aid future studies investigating type 2 diabetes (10). Furthermore, miRNA microarray analysis has revealed that miR-4530 is also upregulated in enterovirus 71-infected cells. Further investigation into the prediction of miR-4530 target genes may aid future studies investigating the regulatory mechanism of miRNAs underlying the pathogenesis of enterovirus 71 infected cells (42). In conclusion, miR-4530 can be considered to be an important marker for diagnosis and further mechanistic studies should be conducted.

In conclusion, the function of miR-4530 in the enhancement of apoptosis and the suppression of cell growth in HUVECs suggests that miR-4530 possess value in the treatment of cancer. Furthermore, the results of the present study provide a foundation for future clinical drug research. In conclusion, miR-4530 possesses a significant role in cellular functions and may be applicable for use in targeted drug delivery for the treatment of tumors.

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Availability of data and materials

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

Authors' contributions

LZ and JL conceived and designed the experiments, which were performed by LJ and HL. LJ and TZ analyzed the data and wrote the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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