miRNA-92a inhibits vascular smooth muscle cell phenotypic modulation and may help prevent in-stent restenosis

FENFEN JIANG[1,2*], BIN ZHANG[3*], XIANGYU ZHANG[2], RAN ZHANG[2], QIN LU[2], FENGJIE SHI[2], JIANJIANG XU[2] and LANG DENG[1]

[1]Department of Cardiology, Huzhou Central Hospital, Affiliated Central Hospital of Huzhou University, Huzhou, Zhejiang 313003; Departments of [2]Cardiology and [3]Anaesthesiology, The Second Affiliated Hospital of Jiaxing University, Jiaxing, Zhejiang 314000, P.R. China

Received May 9, 2022; Accepted November 23, 2022

DOI: 10.3892/mmr.2023.12927

Abstract. The modulation of vascular smooth muscle cell (VSMC) phenotype during cellular proliferation and migration may represent a potential therapeutic approach for vascular intimal hyperplasia prevention. However, the precise role of this process in VSMC biology and remodeling remains unclear. In the present study, western blotting, PCR, MTT and Transwell assays were used to analyze related protein and mRNA expression, cell viability and cell migration, respectively. It was demonstrated that miR-92a modulated VSMCs into a synthetic phenotype via the Kruppel-like factor 4 (KLF4) pathway. Targeting microRNA (miRNA/miR)-92a in VSMCs using a KLF4 inhibitor suppressed the synthetic phenotype and inhibited VSMC proliferation and migration. To further confirm this finding, the expression levels of miR-92a were measured in patients undergoing coronary artery intervention. The serum miR-92a expression levels were significantly higher in patients with in-stent restenosis (ISR) compared with those in patients without ISR, whereas KLF4 expression was significantly reduced in the non-ISR group. Bioinformatic analysis and promoter-luciferase reporter assays were used to examine the regulatory mechanisms underlying KLF4 expression. KLF4 was demonstrated to be transcriptionally upregulated by miR-92a in VSMCs. miRNA transfection was also performed to regulate the level of miR-92a expression. miR-92a overexpression inhibited VSMC proliferation and migration, and also increased the mRNA and protein expression levels of certain differentiated VSMC-related genes. Finally, miR-92a inhibition promoted the proliferation and migration of VSMCs, which could be reversed using a KLF4 inhibitor. Collectively, these results indicated that the local delivery of a KLF4 inhibitor may act as a novel therapeutic option for the prevention of ISR.

Correspondence to: Dr Lang Deng, Department of Cardiology, Huzhou Central Hospital, Affiliated Central Hospital of Huzhou University, 198 Hongqi Road, Huzhou, Zhejiang 313003, P.R. China
E-mail: langzi213317@126.com

*Contributed equally

Key words: in-stent restenosis, vascular smooth muscle cells, coronary atherosclerotic heart disease, miRNA-92a, Kruppel-like factor 4

Introduction

Coronary atherosclerotic heart disease (CAHD) represents the leading cause of death in the global population (1). The incidence and prevalence of heart disease is expected to rise due to the combination of the rise in obesity and predicted worsening of other cardiovascular risk factors in the general population (2). With recent advancements in revascularization techniques and technologies, percutaneous coronary intervention (PCI) and stenting have become the most commonly performed procedures for the treatment of CAHD (3). First generation drug-eluting stents (DESs) such as the Cypher™ sirolimus eluting stent and Taxus™ paclitaxel eluting stent have led to a radical reduction in restenosis (4). However, in-stent restenosis (ISR) remains a significant burden for patients undergoing coronary intervention (5). Although the introduction of DESs has reduced the rate of target lesion revascularization (TLR) when compared with bare metal stents (BMSs), 7-10% of patients continue to require further procedures within 5 years of treatment, and ~20% of patients need TLR within 10 years (6,7).

ISR is characterized by platelet aggregation, growth factor release, inflammatory cell infiltration, medial smooth muscle cell proliferation and migration, and extracellular matrix remodeling (8). The vascular response to injury is dependent on the type of cells within the vessels and is also mediated by circulating cells derived from the bone marrow (9). Understanding the molecular mechanisms underlying the physiological healing process and the response to pathological restenosis has become the focus for extensive research.

Vascular smooth muscle cells (VSMCs) retain marked levels of plasticity during postnatal development and can undergo dedifferentiation into a synthetic phenotype (10). This process is considered to provide a survival advantage, as it permits the efficient repair of the vasculature following injury (11). Similar to numerous evolutionarily conserved

processes, these properties can also be considered a disadvantage and predispose patients to various abnormal responses after injury, which contribute to restenosis (8). In a previous study, Chen (12) reported that the recruitment of smooth muscle cells into the intima of the vessel wall was a significant contributor to atherosclerotic plaque progression. Moreover, VSMCs within the medial layer of the vessel wall were then activated to migrate and proliferate in response to ISR (12). A previous study suggested that the Kruppel-like factor (KLF) family serves a vital role in homeostasis maintenance within the body, including in the immune, digestive, respiratory, hematopoietic and cardiovascular systems (13). KLF members demonstrate variable biological functions and distinct phenotypes in different diseases, which mainly result from their N-terminal sequences, which provide unique protein interaction motifs and post-translational modification sites (14). KLF4 is expressed in the vascular wall, which includes endothelial cells (ECs) and VSMCs, and has been reported to serve a critical role in vascular wall biology. KLF4 confers an anti-inflammatory and vasoprotective phenotype on ECs by the inhibition of NF-κB activation (15). More importantly, it has been reported that KLF4 inhibits angiogenesis and endothelial proliferation by mediating microRNAs (miRNAs/miRs) in VSMCs (14,16). miRNAs are a class of novel endogenous regulators of gene expression that act at the post-transcriptional level. Furthermore, miRNAs serve essential roles in the regulation of numerous cellular events, including cellular proliferation, differentiation and apoptosis (17).

miR-92a, a member of the miR17-92 cluster, has been reported to be highly expressed in ECs (18). miR-92a has also been reported to serve a potential functional role in VSMCs by providing protection against apoptosis induced by oxidative stress (19). However, little is known about the role of miR-92a and its associated mechanism of action in the phenotypic modulation of VSMCs. In the present study, miR-92a targeting of KLF4 and promotion of VSMC proliferation were evaluated.

Materials and methods

Aortic VSMC culture. Human aortic VSMCs (ZQ0491) were purchased from Shanghai Zhongqiao Xinzhou Biotechnology Co., Ltd. The cells were 3rd generation cells and were cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) supplemented with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.) and 1% penicillin/streptomycin in a humidified atmosphere containing 5% CO_2 at 37˚C. Cells were grown to 80-90% confluence and passaged at a ratio of 1:3. The cells used in the experiments were passaged three to five times before use. Kenpaullone (HY-12302) was used to treat VSMCs at a concentration of 5 μM for 6 h.

Cell transfection. The miR-92a mimic, negative control (NC) mimic, miR-92a inhibitor and NC inhibitor were purchased from Guangzhou RiboBio Co., Ltd., and were transfected into cells using Lipofectamine® 2000 (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. Briefly, cells were seeded in 6-well plates and transfected to ~80% confluence. The related mimic or inhibitor (50 nM) were transfected into cells using Lipofectamine 2000 in OPTI-MEM (Gibco; Thermo Fisher Scientific, Inc.). The cells were cultured for 6-8 h in an incubator at 37˚C, then the transfection medium was discarded and cells were cultured with normal medium for 24 h at 37˚C before use. The sequences of the miRNAs used were as follows: miR-92a mimic forward (F), 5'-UAU UGCACUUGUCCCGGCCUGU-3' and reverse (R), 5'-AGG CCGGGACAAGUGCAAUAUU-3'; mimics NC F, 5'-UUC UCCGAACGUGUCACGUTT-3' and R, 5'-ACGUGACAC GUUCGGAGAATT-3'; miR-92a inhibitor, 5'-ACAGGCCGG GACAAGUGCAAU-3'; and miRNA inhibitor NC, 5'-CAG UACUUUUGUGUAGUACAA-3'.

Patient sample collection and inclusion criteria. The Second Affiliated Hospital of Jiaxing University (Jiaxing, China) provided blood samples from patients with CAHD after PCI. The details of the patients (age range, 45-79 years) [ISR (n=20) and non-ISR (n=20)] are presented in Table I. The blood samples were frozen at -80˚C and stored until RNA extraction. Written informed consent was provided by each patient. All experimental protocols were approved by The Second Affiliated Hospital of Jiaxing University (institutional review board protocol number: JXEY-2021JX083). Patients who visited the Second Affiliated Hospital of Jiaxing University between January 1, 2021, and June 31, 2022, were included. The inclusion criteria were as follows: i) An age of 18-80 years; ii) patient received a DES during the first PCI; iii) patient received chlorine treatment with 75 mg/day clopidogrel and 100 mg/day aspirin; iv) coronary angiography was followed up for 10-12 months; v) arterial embolism or acute myocardial infarction were excluded in patients with complete occlusion; and vi) cardiac function grades I-III. Exclusion criteria were as follows: i) Age <18 or >80 years; ii) myocardial infarction during treatment; iii) failure to take medication regularly for a long time (≥3 months); and iv) cardiac function grade IV. ISR was defined as stenosis of ≥50% in the stent segment and ≥5 mm at the stent edge during follow-up coronary angiography after PCI; non-ISR was defined as <5 mm in the stent segment and <5 mm at the edge of the stent during follow-up coronary angiography, with diameter stenosis <50%.

Western blotting. RIPA buffer containing protease inhibitors (Beyotime Institute of Biotechnology) was used to extract the total protein from cells. For each sample, 30 μg protein (measured with a bicinchoninic acid protein assay kit) was added per lane, separated on 10% gels using SDS-PAGE and transferred onto a 0.45-μm PVDF membrane. After blocking with 5% non-fat milk at 37˚C for 2 h, the membrane was incubated separately at 4˚C overnight with antibodies as follows: SMMHC antibody (1:500; cat. no. 18569-1-AP), α-SMA antibody (1:1,500; cat. no. 14395-1-AP), osteopontin (OPN) polyclonal antibody (1:500 dilution; cat. no. 25715-1-AP) and GAPDH polyclonal antibody (1:8,000, cat. no. HRP-60004) (all ProteinTech Group, Inc.). The following day, the PVDF membrane was washed three times with 0.1% Tween 20 (TBST) containing 5% bovine serum albumin and incubated with goat anti-rabbit HRP (1:2,000; cat. no. SAB90200; Frdbio Bioscience & Technology) and goat anti-mouse HRP (1:8,000; cat. no. SAB90100, Frdbio Bioscience & Technology) secondary antibodies for 2 h at room temperature. The blots were visualized using ECL reagents (cat. no. P0018S; Beyotime

Table I. Baseline characteristics of the study subjects.

Characteristic	Non-ISR (n=20)	ISR (n=20)	P-value
Age, years[a]	61.75±8.43	66.32±8.42	0.099
Sex (male), n	15	15	-
BMI, kg/m^{2}[a]	23.55±1.43	23.94±1.28	0.374
History of smoking, n	9	10	-
Drinking history, n	6	7	-
Diabetes (type 2), n	6	6	-
Hypertension, n	11	12	-
Hyperlipidemia, n	3	4	-
TC, mg/dl[a]	70.75±31.17	78.65±24.37	0.385
TG, mg/dl[a]	29.24±6.53	30.11±7.19	0.694
HDL-C, mg/dl[a]	19.66±6.06	21.02±5.40	0.465
LDL-C, mg/dl[a]	34.63±14.68	38.23±15.31	0.459

[a]Mean ± standard deviation. ISR, in-stent restenosis; BMI, body mass index; TC, total cholesterol; TG, triglyceride; HDL-C, high density lipoprotein-cholesterol; LDL-C, low density lipoprotein-cholesterol.

Institute of Biotechnology). The semi-quantitative analysis of protein bands was performed using ImageJ software V1.8.0 (National Institutes of Health).

Reverse transcription-quantitative PCR (RT-qPCR) assay. Total RNA from patients' blood was extracted using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.) and reverse transcription was performed at 42˚C for 2 min, 37˚C for 15 min and 85˚C for 5 sec to obtain the first strand of complementary DNA using a PrimeScript® RT reagent kit (Takara Biotechnology Co., Ltd.). qPCR was performed using 2X SYBR Green Master Mix (cat. no. S2014; US Everbright Inc.). The thermocycling conditions were as follows: 95˚C for 30 sec, 15 sec at 95˚C and 60 sec at 60˚C for 45 cycles. All mRNA and miRNA expression levels were normalized to GAPDH or U6, respectively, using the $2^{-\Delta\Delta Cq}$ method (20). The primer sequences used were as follows: α-SMA F, 3'-TGTTCCAGCCGTCCTTCATC-5' and R, 3'-GGGAGCCAAAGCAGTGATCT-5'; SMMHC F, 3'-CGAAGGGCTTGAATGAGGAGT-5' and R, 3'-GCTTCCTCCCAAGGAGCTGTAT-5'; OPN F, 3'-GAGGAAAAGGAGACCCTTCCA-5' and R, 3'-TGAAAACTTCGGTTGCTGGC-5'; KLF4 F, 3'-ATGCTCACCCCACCTTCTTC-5' and R, 3'-CTTCCCCTCTTTGGCTTGGG-5'; miR-92a, 3'-TATATCTATTGCACTTGTCCCG-5'; miR-125b, 3'-TCCCTGAGACCCTAACTTGTGA-5'; miR-26a, 3'-GCCGAGTTCAAGTAATCCAGGA-5'; miR-214, 3'-TGCCTGTCTACACTTGCTGTGC-5'; miR-199a, 3'-CCCAGTGTTCAGACTACCTGTTC-5'; miR-9, 3'-GCCGAGTCTTTGGTTATCTAGCT-5'; miR-559, 3'-TCGGCAGGTAAAGTAAATATG-5'; miR-100, 3'-AACCCGTAGATCCGAACTTGTG-5'; U6 F, 3'-CTCGCTTCGGCAGCACA-5' and R, 3'-AACGCTTCACGAATTTGCGT-5'; and GAPDH F, 3'-ACAGTCAGCCGCATCTTCTT-5' and R, 3'-GACTCCGACCTTCACCTTCC-5'. The miR-125b, miR-26a, miR-214, miR-199a, miR-559 and miR-100 downstream primers were universal primers provided in a customized kit [General Biology (Anhui) Co., Ltd.].

Dual-luciferase reporter assay. The relative luciferase activity was detected using a Dual-Luciferase Assay Kit (cat. no E190; Promega Corporation). A bioinformatics analysis performed using TargetScan 7.1 (https://www.targetscan.org/vert_71/) demonstrated that KLF4 contained an miR-92a binding site at the 3'-untranslated region (3'-UTR). The mutant 3'-UTR of KLF4 was amplified using the pGL3/Luc-KLF4 3'-UTR as a template and cloned downstream of the pGL3/Luc vector (Shanghai GenePharma Co., Ltd.). VSMCs were seeded into 96-well plates and co-transfected with either 100 nM miR-92a mimic or NC and 2 μg pGL3/Luc-KLF4 3'-UTR or mutant 3'-UTR using Lipofectamine 2000 reagent (Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol. The relative luciferase activity was determined 48 h later based on the ratio of the luciferase activity of firefly luciferase to that of *Renilla* luciferase. Luciferase intensity was assessed using the Dual Luciferase Reporter 1000 Assay System (Promega Corporation).

MTT assay. VSMCs transfected with miR-92a mimic or inhibitor were seeded into 96-well plates with an adjusted density of 7,000 cells per well and cultured at 37˚C under 5% CO_2 for 24, 48, 72 or 96 h. Next, MTT (MilliporeSigma) diluted in DMEM was added to the medium, with a final concentration of 5 mg/ml in each well for a further 4 h. The supernatant was carefully removed and dimethyl sulfoxide (200 μl) was added to each well. The suspension was placed in the dark for 2 h at room temperature, after which the absorbance was quantified at 570 nm using an absorbance reader (Thermo Fisher Scientific, Inc.).

Transwell migration assays. Cellular migration was assessed using Transwell chambers with a pore size of 8 μm (Corning, Inc.). Briefly, following transfection with an miR-92a mimic or miR-92a inhibitor, 1x10^{5} VSMCs were added to the upper chamber in serum-free DMEM medium and the low chamber was filled with culture medium containing 10% FBS and

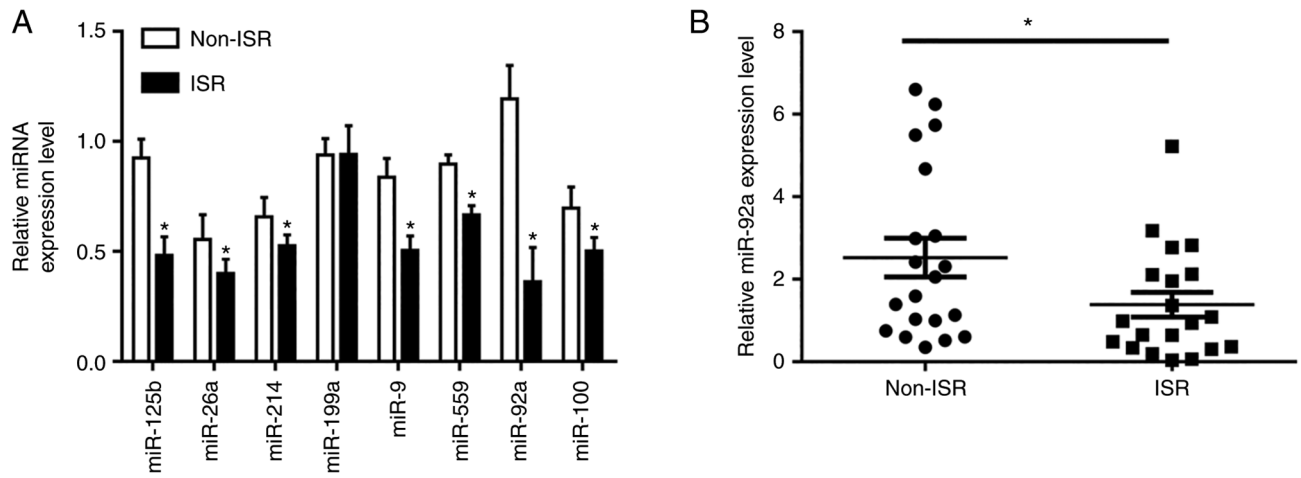

Figure 1. miRNA expression levels in ISR patients. (A) Reverse transcription-quantitative PCR was used to assess the expression levels of miR-125b, miR-26a, miR-214, miR-199a, miR-9, miR-559, miR-92a and miR-100. *P<0.05 vs. non-ISR. (B) miR-92a expression levels were significantly lower in ISR patients (n=20) than in control patients without ISR (n=20). Each point represents the miR-92a expression from an individual patient. *P<0.05. miRNA/miR, microRNA; ISR, in-stent restenosis.

incubated at 37˚C. After 0, 24, 48, 72 or 96 h, the insert membranes were fixed using chilled methanol and stained using 0.5% crystal violet at room temperature. The number of invading cells was counted under an inverted light microscope and imaged.

Statistical analysis. Results are presented as the mean ± standard deviation or n data. Statistical analysis was performed using SPSS version 13.0 (SPSS, Inc.) and GraphPad Prism 8.0 (GraphPad Software, Inc.). ImageJ software 6.0 (National Institutes of Health) was used to perform image analysis. The statistical significance of the differences between groups was calculated using one-way analysis of variance followed by Tukey's post hoc test, or using a two-tailed unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference. Bioinformatics (http://starbase.sysu.edu.cn/index.php) was used to analyze the relationship between miR-92a and KLF4. Pearson's correlation was used to analyze the linear relationship between miR-92a and KLF4. All experiments were repeated three times unless otherwise stated.

Results

Association between miR-92a and KLF4 in patients with ISR. To assess the miRNA expression levels in ISR and non-ISR patients, RT-qPCR was used to assess the expression levels of certain serum miRNAs of interest in blood samples from ISR and non-ISR patients. In the preliminary experiment, certain existing miRNAs were used to assess the difference between ISR and non-ISR groups, which demonstrated that the expression levels of miR-125b, miR-26a, miR-214, miR-9, miR-559, miR92a and miR-100 were significantly different. The results demonstrated that miR-92a was the miRNA with the greatest difference between the ISR and non-ISR groups (Fig. 1A); therefore, it was used in subsequent experiments. Further experiments demonstrated that the expression levels of miR-92a were significantly lower in the patients with ISR (n=20) compared with the control (non-ISR) patients (n=20) (Fig. 1B). However, as presented in Table I, the comorbidities and lipid level combined indices in non-ISR and ISR groups were not significantly different. A schematic representation of the current study is shown in Fig. S1. These results indicated that miR-92a was downregulated in ISR.

miR-92a promotes KLF4 expression by targeting its 3'-UTR. Previous studies have reported that KLF4 is an antiproliferative regulator of VSMCs (21). In the present study, to elucidate whether KLF4 was a direct target of miR-92a, a bioinformatics approach was used to search for a potential miR-92a matching site in the KLF4 3'-UTR (Fig. 2A). The level of KLF4 was upregulated in the ISR group (Fig. 2B). A luciferase reporter assay was used to determine the interaction between KLF4 and miR□455-5p (Fig. 2C). It was also demonstrated that miR-92a levels were significantly inversely associated with the mRNA expression level of KLF4 in patients with ISR, as demonstrated using a Pearson's correlation model (Fig. 2D). This suggested that this miRNA served an important role in the regulation of gene expression.

miR-92a contributes to cerebral VSMC synthetic phenotype switching. To evaluate whether miR-92a modulated the VSMC phenotype, the mRNA and protein expression levels of contractile and synthetic protein markers in cerebral VSMCs were assessed in the present study. In both mature and normal blood vessels, VSMCs possess a highly quiescent and contractile phenotype that is associated with high levels of contractile marker proteins, such as α-smooth muscle actin (α-SMA) and smooth muscle myosin heavy chain (SMMHC) (22). In atherosclerosis and arterial restenosis, VSMCs can change to a dedifferentiated, proliferative and migratory phenotype via the downregulation of the gene expression of VSMC contractile markers and via the upregulation of OPN synthetic protein expression (23,24). First, the level of miR-92a was confirmed after transfection with miR-92a mimic or inhibitor (Fig. 3A). Compared with those in the NC group, the mRNA and protein expression

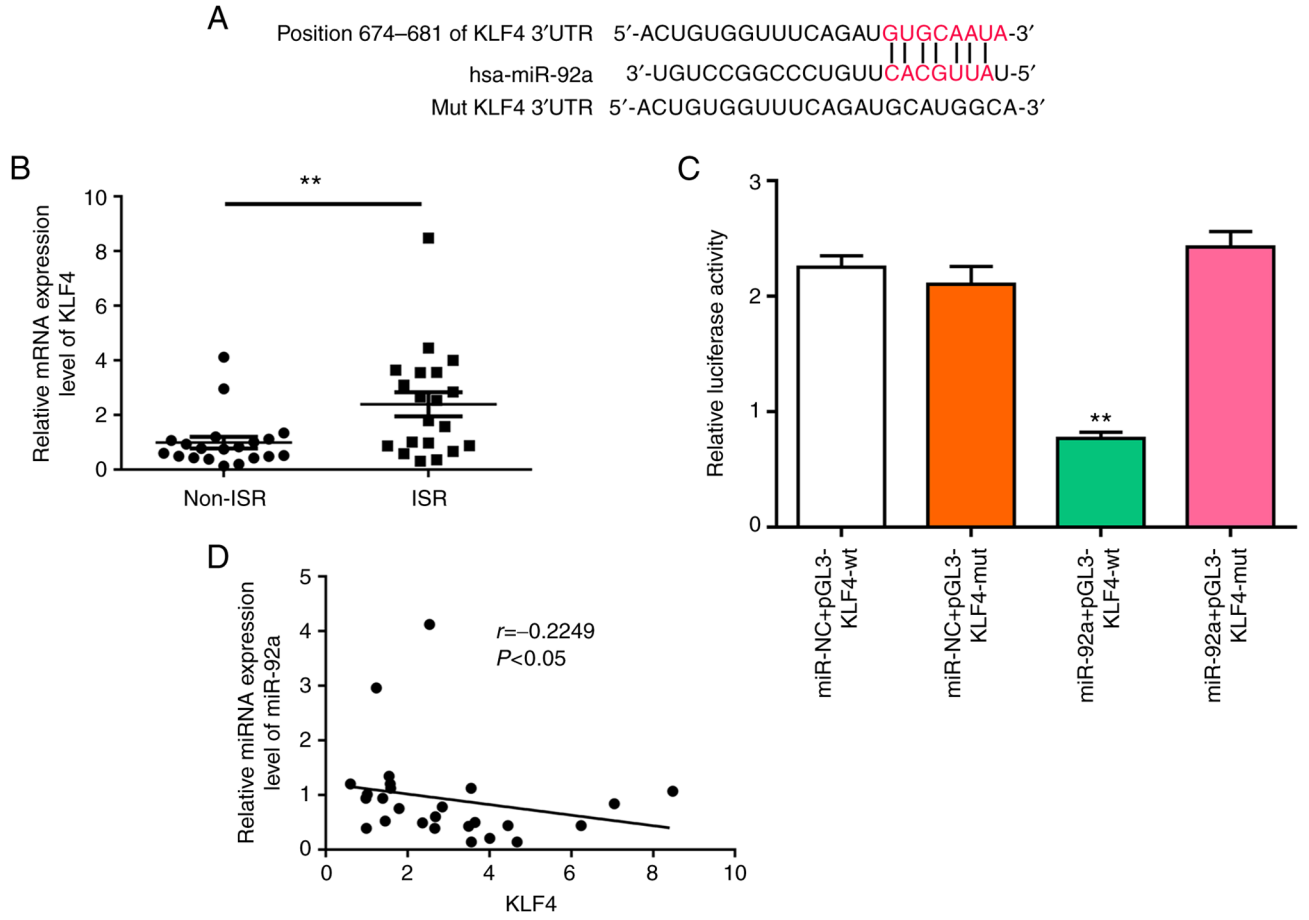

Figure 2. miR-92a promotes KLF4 expression by targeting its 3'-UTR. (A) The binding site for miR-92a was located at nucleotides 674-681 of the *Homo sapiens* KLF4 mRNA 3'-UTR. (B) The mRNA expression level of KLF4 was higher in ISR patients (n=20) than in control subjects without ISR (n=20). **P<0.01. (C) VSMCs were co-transfected with miR-92a or miR-NC and pGL3-KLF4-wt or mutant pGL3-KLF4-mt. After 24 h, luciferase activity was assessed. **P<0.01. n=3 for each group. (D) miR-92a expression levels were negatively associated with KLF4 mRNA expression levels in ISR patients. miR, microRNA; UTR, untranslated region; KLF4, Kruppel like factor 4; VSMC, vascular smooth muscle cell; wt, wild-type; mt, mutant; ISR, in-stent restenosis.

levels of α-SMA and SMMHC were significantly lower in the miR-92a inhibitor group (Fig. 3B, C and E-G). However, the mRNA and protein expression levels of OPN were significantly higher in the miR-92a inhibitor group compared with those in the NC (Fig. 3D and H). Collectively, these results demonstrated that miR-92a could promote VSMC phenotypic modulation.

miR-92a promotes VSMC phenotypic modulation. To evaluate the functional effects of miR-92a, VSMCs were transfected with an miR-92a mimic or inhibitor for 24 h and assessed using MTT and Transwell migration analysis. The results demonstrated that compared with the NC, treatment with an miR-92a inhibitor could significantly increase cell proliferation, whereas the miR-92a mimic significantly reduced proliferation compared with the NC (Fig. 4A). Furthermore, the number of migrating cells was markedly increased after treatment with the miR-92a inhibitor compared with the number in the NC group (Fig. 4B).

Kenpaullone (HY-12302) promotes synthetic phenotype switching in cerebral VSMCs. We hypothesized that KLF4 may partially mediate the effects of miR-92a on VSMCs. HY-12302 is a small molecule inhibitor of KLF4, which is used in conjunction with miR-92a inhibitors in functional assays involving VSMCs. In the present study, VSMCs demonstrated significantly increased expression of the synthetic gene, OPN, following transfection with an miR-92a inhibitor, and significantly decreased expression of the contractile genes (α-SMA and SMMHC) at both the mRNA (Fig. 5A-C) and protein (Fig. 5D-G) expression levels compared with the NC. These results demonstrated that treatment with the miR-92a inhibitor promoted a phenotypic alteration in VSMCs from differentiated to de-differentiated cells. Moreover, treatment with the KLF4 inhibitor, HY-12302, significantly antagonized the de-differentiation response of VSMCs to the miR-92a inhibitor at the mRNA and protein expression levels (Fig. 5).

HY-12302 partially abrogates the migration and proliferation of VSMCs. The MTT and Transwell migration assays were used to evaluate the VSMC cell proliferation and migration capabilities of each group. The KLF4 inhibitor, HY-12302, significantly abrogated the accelerated cellular proliferation induced by treatment with the miR-92a inhibitor. Furthermore, treatment with HY-12302 combined with the miR-92a inhibitor was demonstrated to markedly reduce cellular migration compared with the miR-92a inhibitor group (Fig. 6).

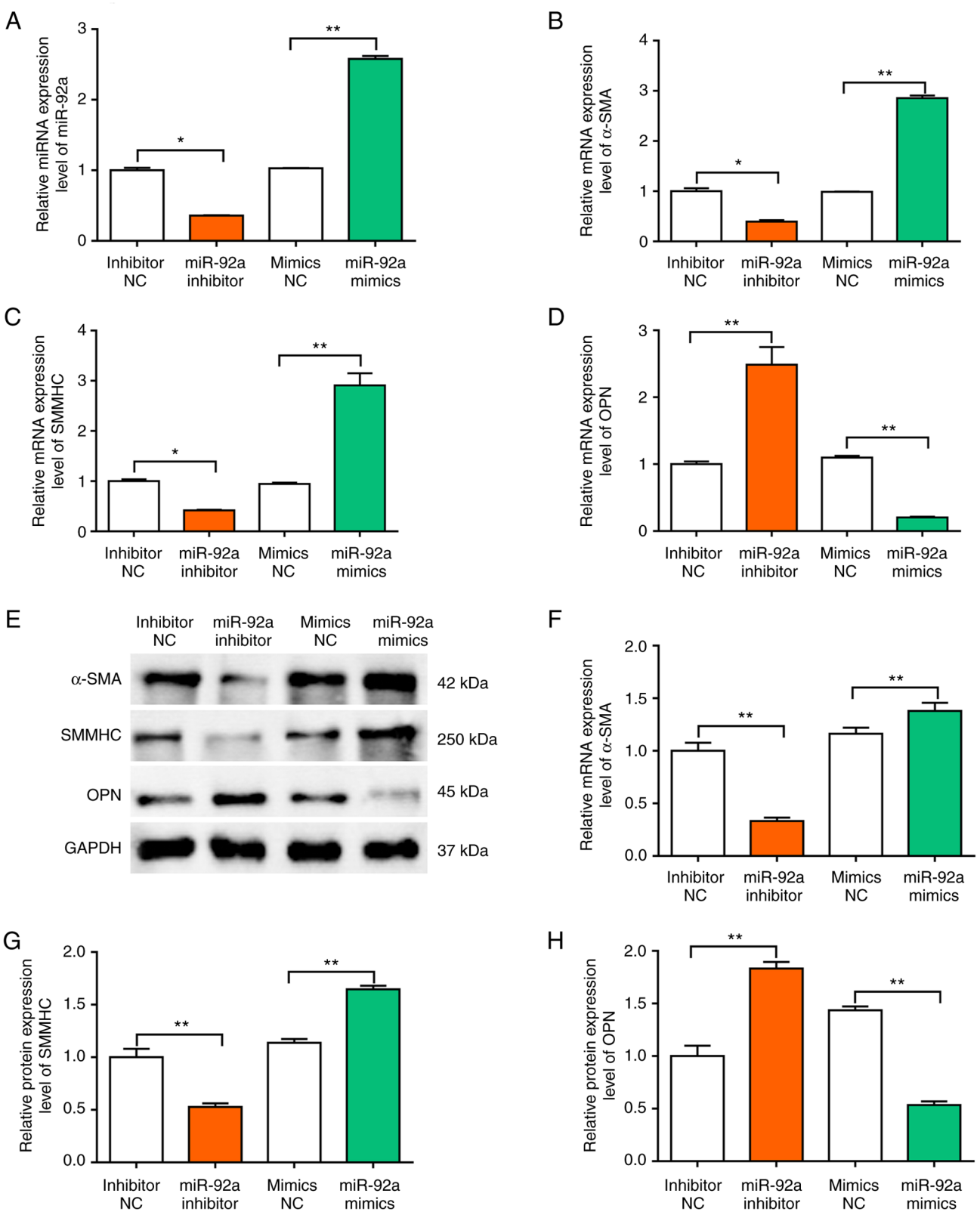

Figure 3. miR-92a contributes to cerebral VSMC synthetic phenotype switching. (A) The expression level of miR-92a in VSMCs transfected with an miR-92a mimic or inhibitor. (B-D) mRNA and (E-H) western blotting of VSMC contractile and synthetic markers. *P<0.05 and **P<0.01; n=3. miRNA/miR, microRNA; VSMC, vascular smooth muscle cell; NC, negative control; α-SMA, α-smooth muscle actin; SMMHC, smooth muscle myosin heavy chain; OPN, osteopontin.

Discussion

Revascularization remains the cornerstone for the management of patients with CAHD. Although PCI and metal scaffold insertion, known as stenting, has become the preferred method for the restoration of vessel patency, up to 30% of patients will gradually experience a re-narrowing of the lumen caused by neointima formation, which results in a condition known

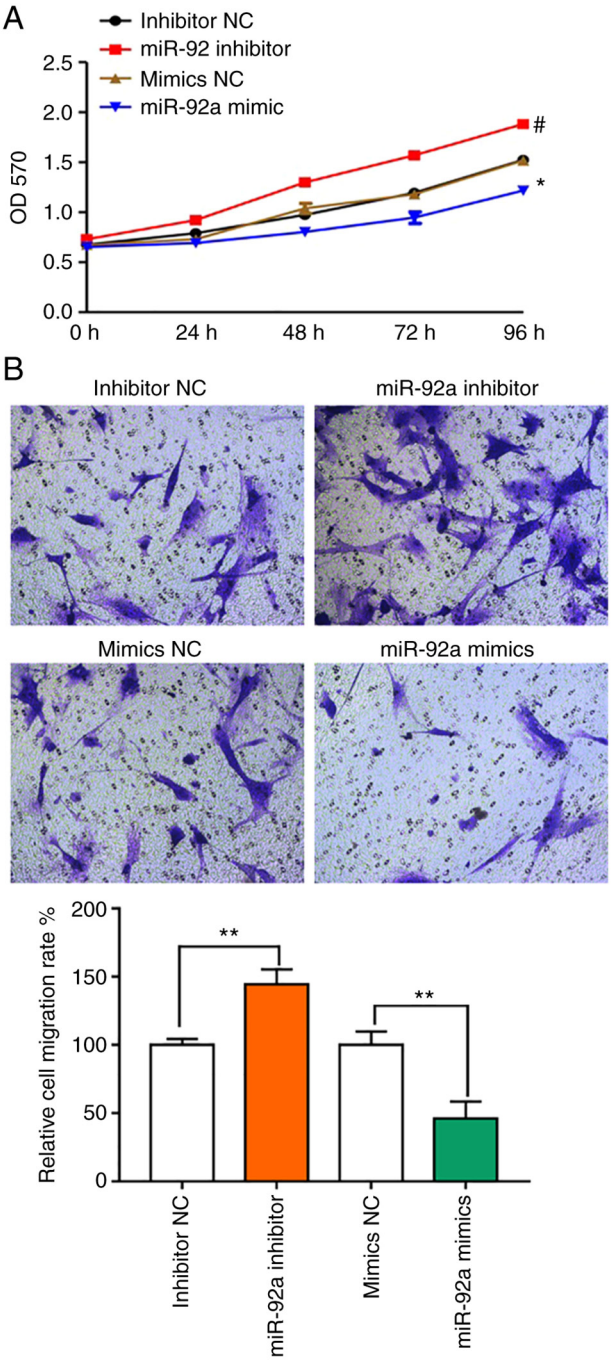

Figure 4. miR-92a promotes VSMC phenotypic modulation. (A) VSMC proliferation was evaluated using an MTT assay in VSMCs transfected with an miR-92a mimic or inhibitor. *P<0.05 vs. mimic NC; $^{\#}$P<0.05 vs. inhibitor NC. (B) VSMC migration was assessed using a Transwell assay in VSMCs transfected with an miR-92a mimic or inhibitor. **P<0.01. miR, microRNA; VSMC, vascular smooth muscle cell; NC, negative control; OD, optical density.

as ISR (25). Descriptive histological studies reported that ISR involved an influx of inflammatory cells subsequent to the appearance of synthetic SMCs and myofibroblasts that produced an abundant extracellular matrix (ECM) (26,27). In response to vascular injury, growth factors and inflammatory stimuli, VSMCs can switch from a quiescent 'contractile' phenotype into an active 'synthetic' phenotype (28). Fully differentiated or mature VSMCs have been reported to exhibit the contractile phenotype, with characteristics of a very low rate of proliferation and the expression of certain contractile proteins, such as SMMHC, α-SMA and calponin (29). Moreover, while the proteins are all necessary for contractile functionality, upon vascular injury (such as caused by angioplasty or bypass surgery), VSMCs can de-differentiate from the contractile phenotype to a highly synthetic phenotype (30). This new phenotype is characterized by higher proliferation and migration rates, enhanced ECM component production and decreased VSMC-specific markers (31). Synthetic VSMCs migrate and proliferate, and their accumulation over time leads to the formation of the neointima (32,33). As such, the inhibition of inflammation in VSMCs may represent an important step in limiting vascular injury-triggered stenosis in a clinical scenario. Consistent with this hypothesis, the present study demonstrated significantly increased mRNA and protein expression levels of VSMC synthetic proteins and proliferation in the presence of miR-92a mimics. These results demonstrated that miR-92a was a positive regulator of the VSMC synthetic phenotype *in vitro*. The first limitation of the present study was that certain existing miRNAs previously purchased by the research group were used to assess the difference between ISR and non-ISR, and that these 8 miRNA had a marked difference in ISR and non-ISR samples; therefore, the effect of miR-92a in ISR was demonstrated accidentally.

KLF4 serves an important role in a number of vascular diseases and is expressed by a range of cell types involved in vascular disease development, including VSMCs (34). One previous study reported KLF4 as a transcriptional target that could modulate VSMC differentiation (35). Although KLF4 is not typically expressed in differentiated VSMCs *in vivo*, it is transiently induced in VSMCs following vascular injury (36). The present study demonstrated that the protein expression level of KLF4 expression was significantly higher in ISR patients, whereas the levels of miR-92a expression were significantly lower. Other information about the comorbidities and the lipid level combined indices in non-ISR and ISR was evaluated; however, there were no significant differences between these two groups (Table I). Qiu and Sun (37) reported that the number of lesions, MALAT1 expression level, diabetes, N-terminal pro-brain natriuretic peptide and high sensitivity C-reactive protein were independent risk factors for ISR, in 95 patients with coronary heart disease who presented to The Second Hospital of Shandong University (Jinan, China); however, the data from the present study only demonstrated that miR-92a and KLF4 expression were associated with ISR. It could be hypothesized that this difference may be due to the lack of different geographical cases. Furthermore, the evaluation of more cases from different areas could more comprehensively reflect the differences in relevant indicators between ISR patients and non-ISR patients, which was the second limitation of the present study.

The present study demonstrated that the miRNA expression level of miR-92a was significantly negatively associated with the KLF4 protein expression level. Collectively, the data from the present study indicated that KLF4 was a direct target of miR-92a. This suggested that specific mediation of the VSMC-miR-92a-KLF4 pathway may represent an effective therapeutic option by which ISR can be mitigated in the clinic. miRNAs are a class of non-coding small RNA molecules that serve an important role in regulating

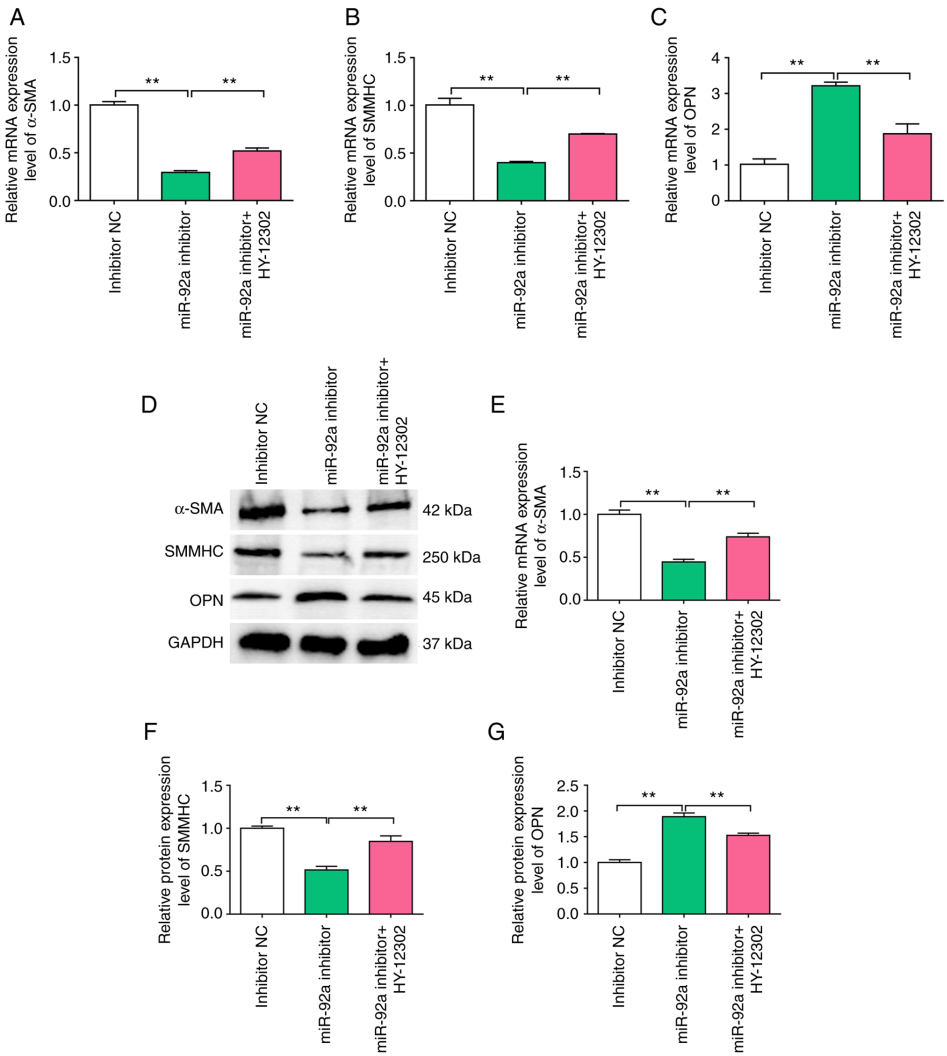

Figure 5. HY-12302 abrogates miR-92a inhibitor-induced VSMC de-differentiation. (A-C) Relative mRNA expression levels of α-SMA, SMMHC and OPN. (D) Western blotting was used to assess the protein expression levels of contractile proteins (α-SMA and SMMHC) and OPN synthetic protein expression. (E-G) Semi-quantified relative protein expression levels of α-SMA, SMMHC and OPN. **P<0.01; n=3. miR, microRNA; α-SMA, α-smooth muscle actin; SMMHC, smooth muscle myosin heavy chain; OPN, osteopontin; NC, negative control.

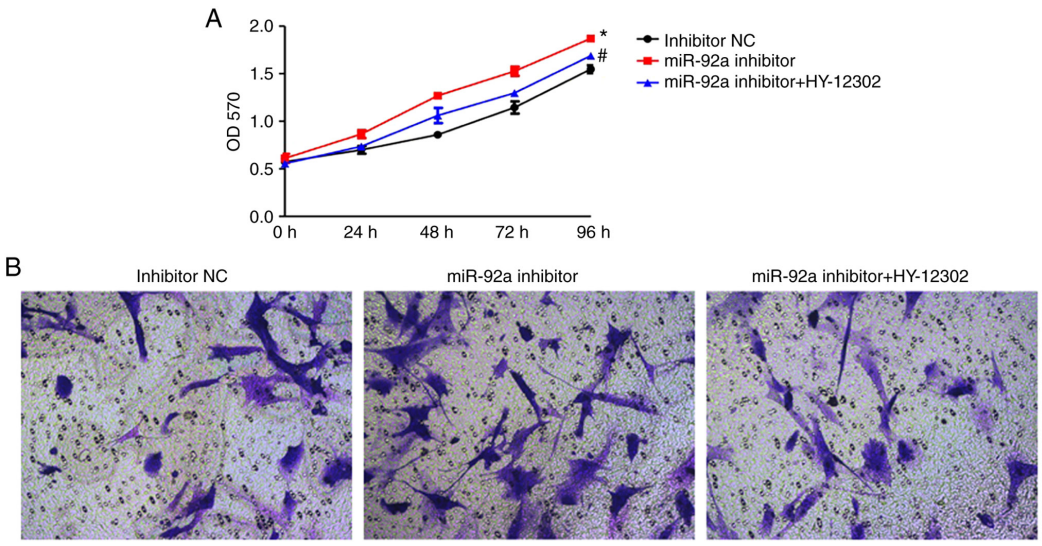

Figure 6. VSMC proliferation and migration is abrogated by KLF4 inhibition. (A) The suppressive effect elicited by the miR-92a inhibitor on cellular proliferation was alleviated by HY-12302. Cell proliferation was monitored at 0, 24, 48, 72 and 96 h using an MTT assay. *P<0.05 vs. inhibitor NC; $^{\#}$P<0.05 vs. miR-92a inhibitor. (B) Representative results of the Transwell plates demonstrating the effects of the miR-92a inhibitor and HY-12302 on VSMC migration compared with the inhibitor NC. VSMC, vascular smooth muscle cell; NC, negative control; miR, microRNA; OD, optical density.

post-transcriptional gene expression. In the majority of cases, miRNAs interact with the 3'-UTR of target mRNAs to inhibit gene translation or induce mRNA degradation (38). Moreover, miR-92a has been reported to be an oncogene in numerous different forms of tumors (39). It was also reported that miR-92a was a negative regulator of endothelial function and angiogenesis (40); however, data from the present study demonstrated that miR-92a was a positive regulator of the synthetic phenotype in VSMCs, functioning by modulation of KLF4. The third limitation of the present study was that the relevant disease model was not established in animal experiments *in vivo* to further investigate the effect of the miR-92A-KLF4 pathway on ISR treatment, which is something that should be evaluated in the future.

In conclusion, the present study demonstrated that the VSMC-miR-92a-KLF4 pathway could mediate the negative regulation of VSMC differentiation in ISR. Since vascular injury induces KLF4 expression and activation, miR-92a overexpression or treatment with a KLF4 inhibitor (HY-12302) could protect against injury-induced VSMC polarization. These effects may be attributed to increased contractile protein expression in VSMCs and the inhibition of proliferation and migration. Given that the expression levels of miR-92a were significantly reduced in ISR patients, the results of the present study provide an important mechanistic foundation to therapeutically target ISR using a KLF4 inhibitor.

Acknowledgements

Not applicable.

Funding

The present study was supported by the Jiaxing Science and Technology Project (grant nos. 2021AD30103, 2020AD30119 and 2021AD30090).

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

FJ and LD conceptualized and planned the study. BZ, XZ and RZ performed the experiments. QL, FS and JX analyzed the data. LD wrote the manuscript. FJ and LD confirm the authenticity of all the raw data. All authors have read and approved the final version of the manuscript.

Ethics approval and consent to participate

Written informed consent was provided by each patient. All experimental protocols were approved by The Second Affiliated Hospital of Jiaxing University (institutional review board protocol number: JXEY-2021JX083).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References

1. Malakar AK, Choudhury D, Halder B, Paul P, Uddin A and Chakraborty S: A review on coronary artery disease, its risk factors, and therapeutics. J Cell Physiol 234: 16812-16823, 2019.
2. Faroux L, Guimaraes L, Wintzer-Wehekind J, Junquera L, Ferreira-Neto AN, Del Val D, Muntané-Carol G, Mohammadi S, Paradis JM and Rodés-Cabau J: Coronary artery disease and transcatheter aortic valve replacement: JACC state-of-the-art review. J Am Coll Cardiol 74: 362-372, 2019.
3. Al-Lamee RK, Nowbar AN and Francis DP: Percutaneous coronary intervention for stable coronary artery disease. Heart 105: 11-19, 2019.
4. Davis AA and Patel VG: The role of PD-L1 expression as a predictive biomarker: An analysis of all US food and drug administration (FDA) approvals of immune checkpoint inhibitors. J Immunother Cancer 7: 278, 2019.
5. Ali RM, Abdul Kader MASK, Wan Ahmad WA, Ong TK, Liew HB, Omar AF, Mahmood Zuhdi AS, Nuruddin AA, Schnorr B and Scheller B: Treatment of coronary drug-eluting stent restenosis by a sirolimus- or paclitaxel-coated balloon. JACC Cardiovasc Interv 12: 558-566, 2019.
6. Lansky A, Grubman D and Scheller B: Paclitaxel-coated balloons: A safe alternative to drug-eluting stents for coronary in-stent restenosis. Eur Heart J 41: 3729-3731, 2020.
7. Ullrich H, Olschewski M, Münzel T and Gori T: Coronary in-stent restenosis: Predictors and treatment. Dtsch Arztebl Int 118: 637-644, 2021.
8. Huynh DTN and Heo KS: Role of mitochondrial dynamics and mitophagy of vascular smooth muscle cell proliferation and migration in progression of atherosclerosis. Arch Pharm Res 44: 1051-1061, 2021.
9. Majesky MW: Vascular development. Arterioscler Thromb Vasc Biol 38: e17-e24, 2018.
10. Touyz RM, Alves-Lopes R, Rios FJ, Camargo LL, Anagnostopoulou A, Arner A and Montezano AC: Vascular smooth muscle contraction in hypertension. Cardiovasc Res 114: 529-539, 2018.
11. Wang Y, Nanda V, Direnzo D, Ye J, Xiao S, Kojima Y, Howe KL, Jarr KU, Flores AM, Tsantilas P, *et al*: Clonally expanding smooth muscle cells promote atherosclerosis by escaping efferocytosis and activating the complement cascade. Proc Natl Acad Sci USA 117: 15818-15826, 2020.
12. Chen MF: The role of calmodulin and calmodulin-dependent protein kinases in the pathogenesis of atherosclerosis. Tzu Chi Med J 34: 160-168, 2021.
13. Fan Y, Lu H, Liang W, Hu W, Zhang J and Chen YE: Krüppel-like factors and vascular wall homeostasis. J Mol Cell Biol 9: 352-363, 2017.
14. Wang H, Xu H, Lyu W, Xu Q, Fan S, Chen H, Wang D, Chen J and Dai J: KLF4 regulates TERT expression in alveolar epithelial cells in pulmonary fibrosis. Cell Death Dis 13: 435, 2022.
15. Rane MJ, Zhao Y and Cai L: Krüppel-like factors (KLFs) in renal physiology and disease. EBioMedicine 40: 743-750, 2019.
16. Li YZ, Wen L, Wei X, Wang QR, Xu LW, Zhang HM and Liu WC: Inhibition of miR-7 promotes angiogenesis in human umbilical vein endothelial cells by upregulating VEGF via KLF4. Oncol Rep 36: 1569-1575, 2016.
17. Du M, Espinosa-Diez C, Liu M, Ahmed IA, Mahan S, Wei J, Handen AL, Chan SY and Gomez D: miRNA/mRNA co-profiling identifies the miR-200 family as a central regulator of SMC quiescence. iScience 25: 104169, 2022.
18. Sand M, Hessam S, Amur S, Skrygan M, Bromba M, Stockfleth E, Gambichler T and Bechara FG: Expression of oncogenic miR-17-92 and tumor suppressive miR-143-145 clusters in basal cell carcinoma and cutaneous squamous cell carcinoma. J Dermatol Sci 86: 142-148, 2017.
19. Zhang L, Zhou M, Wang Y, Huang W, Qin G, Weintraub NL and Tang Y: miR-92a inhibits vascular smooth muscle cell apoptosis: Role of the MKK4-JNK pathway. Apoptosis 19: 975-983, 2014.

20. Livak KJ and Schmittgen TD: Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) method. Methods 25: 402-408, 2001.
21. Li Y, Chen F, Guo R, Jia S, Li W and Zhang B: Tanshinone IIA inhibits homocysteine-induced proliferation of vascular smooth muscle cells via miR-145/CD40 signaling. Biochem Biophys Res Commun 522: 157-163, 2020.
22. Tang HX, Qin XP and Li J: Role of the signal transducer and activator of transcription 3 protein in the proliferation of vascular smooth muscle cells. Vascular 28: 821-828, 2020.
23. Bennett MR, Sinha S and Owens GK: Vascular smooth muscle cells in atherosclerosis. Circ Res 118: 692-702, 2016.
24. Lu QB, Wan MY, Wang PY, Zhang CX, Xu DY, Liao X and Sun HJ: Chicoric acid prevents PDGF-BB-induced VSMC dedifferentiation, proliferation and migration by suppressing ROS/NFκB/mTOR/P70S6K signaling cascade. Redox Biol 14: 656-668, 2018.
25. Kimura Y, Izumiya Y, Araki S, Yamamura S, Hanatani S, Onoue Y, Ishida T, Arima Y, Nakamura T, Yamamoto E, *et al*: Sirt7 deficiency attenuates neointimal formation following vascular injury by modulating vascular smooth muscle cell proliferation. Circ J 85: 2232-2240, 2021.
26. Wang DS, Ganaha F, Kao EY, Lee J, Elkins CJ, Waugh JM and Dake MD: Local stent-based release of transforming growth factor-β1 limits arterial in-stent restenosis. J Lab Autom 21: 305-311, 2016.
27. Osherov AB, Gotha L, Cheema AN, Qiang B and Strauss BH: Proteins mediating collagen biosynthesis and accumulation in arterial repair: Novel targets for anti-restenosis therapy. Cardiovasc Res 91: 16-26, 2011.
28. Sorokin V, Vickneson K, Kofidis T, Woo CC, Lin XY, Foo R and Shanahan CM: Role of vascular smooth muscle cell plasticity and interactions in vessel wall inflammation. Front Immunol 11: 599415, 2020.
29. Pan D, Liu G, Li B, Jiang J, Chen W, Li W, Zhang L, Hu Y, Xie S and Yang H: MicroRNA-1246 regulates proliferation, invasion, and differentiation in human vascular smooth muscle cells by targeting cystic fibrosis transmembrane conductance regulator (CFTR). Pflugers Arch 473: 231-240, 2021.
30. Wu M, Liu W, Huang H, Chen Z, Chen Y, Zhong Y, Jin Z, Liu X and Zou L: PVT1/miR-145-5p/HK2 modulates vascular smooth muscle cells phenotype switch via glycolysis: The new perspective on the spiral artery remodeling. Placenta 130: 25-33, 2022.
31. Shi J, Yang Y, Cheng A, Xu G and He F: Metabolism of vascular smooth muscle cells in vascular diseases. Am J Physiol Heart Circ Physiol 319: H613-H631, 2020.
32. Wu JH, Zhou YF, Hong CD, Chen AQ, Luo Y, Mao L, Xia YP, He QW, Jin HJ, Huang M, *et al*: Semaphorin-3A protects against neointimal hyperplasia after vascular injury. EBioMedicine 39: 95-108, 2019.
33. Wu W, Zhang W, Choi M, Zhao J, Gao P, Xue M, Singer HA, Jourd'heuil D and Long X: Vascular smooth muscle-MAPK14 is required for neointimal hyperplasia by suppressing VSMC differentiation and inducing proliferation and inflammation. Redox Biol 22: 101137, 2019.
34. Wen Y, Lu X, Ren J, Privratsky JR, Yang B, Rudemiller NP, Zhang J, Griffiths R, Jain MK, Nedospasov SA, *et al*: KLF4 in macrophages attenuates TNFα-mediated kidney injury and fibrosis. J Am Soc Nephrol 30: 1925-1938, 2019.
35. Yap C, Mieremet A, de Vries CJM, Micha D and de Waard V: Six shades of vascular smooth muscle cells illuminated by KLF4 (Krüppel-like factor 4). Arterioscler Thromb Vasc Biol 41: 2693-2707, 2021.
36. Ghaleb AM and Yang VW: Krüppel-like factor 4 (KLF4): What we currently know. Gene 611: 27-37, 2017.
37. Qiu S and Sun J: lncRNA-MALAT1 expression in patients with coronary atherosclerosis and its predictive value for in-stent restenosis. Exp Ther Med 20: 129, 2020.
38. O'Brien J, Hayder H, Zayed Y and Peng C: Overview of MicroRNA biogenesis, mechanisms of actions, and circulation. Front Endocrinol (Lausanne) 9: 402, 2018.
39. Li M, Peng J, Shi Y and Sun P: miR-92a promotes progesterone resistance in endometriosis through PTEN/AKT pathway. Life Sci 242: 117190, 2020.
40. Liu PJ, Ye YX, Wang YX, Du JX, Pan YH and Fang XB: MiRNA-92a promotes cell proliferation and invasion through binding to KLF4 in glioma. Eur Rev Med Pharmacol Sci 23: 6612-6620, 2019.