European Review for Medical and Pharmacological Sciences 2019; 23: 2216-2222

Effects of MiR-21 on the proliferation and migration of vascular smooth muscle cells in rats with atherosclerosis via the Akt/ERK signaling pathway

P. SUN[1], L.-N. TANG[2], G.-Z. LI[3], Z.-L. XU[4], Q.-H. XU[1], M. WANG[3], L. LI[4]

[1]Department of Cadre Health Protection, Liaocheng People's Hospital, Liaocheng, China
[2]Department of Critical Care Medicine, Liaocheng People's Hospital, Liaocheng, China
[3]Department of Internal Neurology, Liaocheng People's Hospital, Liaocheng, China
[4]Department of Cardiology, Liaocheng People's Hospital, Liaocheng, China

Abstract. – OBJECTIVE: To explore the effects and mechanism of action of micro ribonucleic acid (miR)-21 on the proliferation and migration of vascular smooth muscle (VSM) in atherosclerosis (AS).

MATERIALS AND METHODS: The rats were fed with a high-fat diet, and the oil red staining was adopted to compare AS between Sprague Dawley (SD) rats and miR-21 knockdown rats. At the *in-vitro* level, primary rat VSM cells (VSMCs) were selected and divided into miR-NC blank control group [miR-normal control (NC) group] and miR-21 overexpression group (miR-21 group) for relevant experimental detection. Wound healing assay and transwell assay were used to detect the effects of miR-21 on the proliferation and migration of VSMCs. Westernn blotting was applied to examine the changes in the levels of Cyclin D, a cell cycle-related protein, and the key factors of the Akt/ERK signaling pathway, such as phosphorylated-Akt (p-AKT), AKT, p-ERK1/2, and ERK1/2. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cell activity assay kit was applied to determine the effects of miR-21 on the proliferation of VSMCs through regulating the Akt/ERK signaling pathway after the ERK signaling pathway inhibitor PD98059 and AKT inhibitor MK-2206 were given.

RESULTS: Compared with that in miR-NC group, the level of AS in miR-21 knockdown rats were decreased significantly ($p < 0.05$). In the cell-level experiment, the overexpression of miR-21 promoted abnormal proliferation of VSMCs and activated the Akt/ERK signaling pathway ($p < 0.05$). MTT assay results revealed that inhibiting the Akt/ERK pathway could reverse the effects of miR-21 promoting proliferation and migration.

CONCLUSIONS: MiR-21 promotes the proliferation and migration of VSMCs by activating the Akt/ERK pathway and aggravates AS. Knocking down miR-21 or inhibiting the Akt/ERK pathway can suppress the activation of VSMCs.

Key Words:
MiR-21, Atherosclerosis, Vascular smooth muscle cells, Akt/ERK signaling pathway.

Introduction

Atherosclerosis (AS) is a chronic inflammation of the arterial wall caused by unbalanced lipid metabolism and inappropriate immune responses. The formation of lesions increases the thickness of the vessel wall, resulting in local oxygen exchange restriction[1,2]. During the progression of AS, the transformation of vascular smooth muscle cells (VSMCs) from static contraction phenotype to proliferative migration phenotype in plaque areas to form fibrous caps is considered to be a basic step in the formation of stable plaques[3]. The two main subtypes of VSMCs include fully differentiated cells responsible for vasoconstriction and relaxation, and migrating proliferative cells activated during growth or injury[4]. In a mouse model of AS, it is found that a single existing smooth muscle cell proliferates to cover the top of the plaque, continues to proliferate and invade the center of the plaque, and forms the plaque together with other cells (such as foam cells)[5]. The proliferation of activated VSMCs contributes to the repair of the vessel wall. However, abnormal regulation of arterial VSMCs leads to the de-differentia-

Corresponding Author: Lin Li, MM; e-mail: 75106595@qq.com

tion of VSMCs and the increase in plaque ECM formation in plaque areas[6]. The research has shown that pathological angiogenesis is involved in the formation and development of vulnerable plaques. At present, pathological angiogenesis is considered as the most powerful independent predictor of the plaque rupture[7].

In the past decade, micro ribonucleic acids (miRNAs) have been found to be involved in the regulations on a series of cellular effects and molecular pathways in AS. Many studies have revealed the importance of miRNAs in key signaling and liposomal homeostasis pathways, and it is a potential regulatory factor for carotid stenosis and AS plaques. Although miR-21 is related to a series of vascular mechanical injury models, its role in the formation of AS remains to be determined[8]. In this work, the effects of miR-21 on the proliferation of VSMCs in atherosclerotic rats were observed through experiments, and its mechanism was preliminarily discussed.

Materials and Methods

Laboratory Animals

MiR-21 knockdown rats were purchased from Shanghai Genechem Co, Ltd. (Shanghai, China).

Laboratory Instruments

The pipettes were purchased from Dragon Laboratory Instruments Ltd. (Beijing, China), low-temperature and high-speed centrifuges from Thermo Scientific (Waltham, MA, USA), the ultra-pure water plant from Millipore (Billerica, MA, USA), the ultra-low temperature refrigerator from Haier (China), electronic balance AE-2000 from Shimizu (Tokyo, Japan), the carbon dioxide incubator from Thermo Scientific (Waltham, MA, USA), the inverted microscope from Olympus (Tokyo, Japan), and the clean bench from Taichang Laboratory Equipment (Jinan, China).

Main Reagents

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Biological Industries (Kibbutz Beit Haemek, Israel), trypsin from Beyotime (Shanghai, China), Lipofectamine 2000 from Invitrogen (Carlsbad, CA, USA), acrylamide gel rapid preparation kit from Sangon (Shanghai, China), and radioimmunoprecipitation assay (RIPA) lysate and dimethyl sulfoxide (DMSO) from Beyotime (Shanghai, China).

Animal Model

Ten clean male healthy Sprague Dawley (SD) rats and 10 miR-21 knockdown rats (miR-21$^{-/-}$), aged 8 weeks old and weighing about 250 g, were selected. They were fed with a high-fat diet (Research Diets) to induce AS and divided into two groups, namely, Control + AS group [normal control (NC) rats fed with a high-fat diet] and miR-21$^{-/-}$ + AS group (miR-21 knockdown rats fed with a high-fat diet). After 10 weeks of feeding, the initial segment of the ascending aorta root was taken, about 1 cm in length and stained with oil red to observe the AS. This study was approved by the Animal Ethics Committee of Liaocheng People's Hospital Animal Center.

Primary Culture of the VSMCs

The rats were euthanized by neck removal, soaked in 75% alcohol and disinfected. The thoracic/abdominal aorta was quickly removed from the biosafety cabinet and washed with phosphate-buffered saline (PBS). The inner and outer membranes of blood vessels and fat were removed from the aorta. Blood vessels were cut into pieces, and primary VMSCs of rats were cultured by tissue block adherence method.

Cell experimental grouping: (1) In miR-21 NC group and miR-21 group, the effects and mechanism of the miR-21 overexpression on the proliferation and migration of VSMCs were verified. (2) PD98059 [extracellular regulated protein kinases (ERK) inhibitor, 25 μmol/L) and MK-2206 [protein kinase B (Akt) inhibitor, 20 μmol/L) were administered to further verify the role of the two channels in mediating miR-21.

Oil Red Staining

After the frozen sections of aortic tissues were dried, the sections were washed with ethanol (50%) slightly and stained with 0.5% oil red solution (0.5 g O + 100 mL 50% ethanol) for 8 min, followed by counterstaining with 5% hematoxylin. After dehydration, mounting, observation and photographing were conducted. Finally, Image-Pro Plus 6.0 (Silver Springs, MD, USA) was used to quantify the AS Area.

Wound Healing Assay

The back of the 6-well plate was scribed at equal intervals using a marker pen and a ruler, with 5 lines/well and about 5×10^5 cells/well, and the cells were incubated overnight. The next day, the cells were scratched out with a 10 μL pipette tip perpendicular to the back horizontal line

of the plate. After the treatment, the cells were transferred into an incubator, and the migration of cells was observed at 0 h and 24 h. Then, EVOS (microscope at the wound site) was applied to take photos for the site with evenly distributed cells and no large number of deaths.

Transwell Assay

After PBS washing, the adherent cells were digested, and the cell suspension was prepared with pure DMEM for later use. The transwell membrane with the pore size of 8 μm was selected for migration determination. 5×10^5 cells were inoculated in each chamber, and the lower chamber was added with 200 μL culture medium containing fetal bovine serum for culture for 36 h. The cells in the chamber were wiped off with alcohol cotton swabs. After that, the chamber was turned over, fixed and stained with crystal violet, followed by observation and photographing. Image-Pro Plus 6.0 was adopted to count the number of cells.

Western Blotting Analysis

RIPA was used to lyse the cells. The adherent cells were scraped off with a spatula, sonicated for 3 times, and centrifuged at 1,3500 rpm/20 min to obtain the protein. Electrophoresis was carried out on 10% SDS-polyacrylamide gel (Sangon, Shanghai, China) with a loading volume of 40 μg/well, and β-actin (Proteintech, Rosemont, IL, USA) was taken as the internal reference. Phosphorylated-Akt (p-Akt), Akt, p-ERK1/2, ERK1/2, and Cyclin D antibodies were purchased from Abcam (Cambridge, MA, USA). Immunoreactivity was detected using the Odyssey imaging system (Biosciences, Franklin Lakes, NJ, USA), and Image-Pro Plus 6.0 software was adopted to quantify Western blotting bands.

Detection of Cell Proliferation Via 3-(4,5)-Dimethylthiazol(-z-y1)-3,5-Diphenyltetrazolium Bromide (MTT)

VSMCs at an appropriate concentration were placed in a 96-well plate, added with medicine and then treated at 24 h, 48 h, and 72 h. Then, the medium was discarded, MTT solution (200 μL/well) (Sigma-Aldrich, St. Louis, MO, USA) was added in the dark for incubation at 37°C for 4 h. 100 μL DMSO was added to each well and shaken in the dark after the supernatant was discarded. Subsequently, the ELx808™ enzyme labeling instrument (BioTek, Biotek Winooski, VT, USA) was applied for detection, and the optical density (OD) value at 570 nm was recorded. The average value of OD of multiple pores indicated the change in the proliferation ability of the corresponding groups of cells.

Statistical Analysis

All the test data were expressed by ($\bar{x} \pm$ SEM) and subjected to one-way analysis of variance. The *t*-test was adopted for the comparison between two groups. $p < 0.05$ represented that the difference was statistically significant.

Results

Detection of the AS Level in Rats

According to the comparison of the oil red staining in ascending aortas between Control + AS group (NC rats fed with a high-fat diet) and miR-21$^{-/-}$ + AS group (miR-21 knockdown rats fed with a high-fat diet), the lipid in the AS area was bright red. It was found that knocking down miR-21 reduced the plaque on the inner wall of blood vessels caused by high-fat feeding and lowered the level of AS, displaying statistical significance (Figure 1).

MiR-21 Promoted the Proliferation and Migration of VSMCs in Rats

MiR-21 was overexpressed in primary rat VSMCs. As shown in Figure 2 (left), wound healing assay revealed that the proliferation and migration of VSMCs were increased in miR-21 overexpression group. As shown in Figure 2 (right), transwell assay and its statistical graphs manifested that miR-21 promoted the migration level of VSMCs. New vessels were involved in the formation and development of vulnerable plaques. Therefore, the high expression of miR-21 could promote the development of AS lesions.

Effects of MiR-21 on the Akt/ERK Signaling Pathway

In order to clarify the mechanism of action of miR-21, Western blotting was adopted to detect the expressions of key proteins of the Akt/ERK signaling pathway and Cyclin D (a cell cycle-related protein). According to the results, the levels of p-Akt and p-ERK1/2 proteins were significantly increased after the overexpression of miR-21, indicating that miR-21 activates the Akt/ERK pathway and promotes the Cyclin D expression (Figure 3).

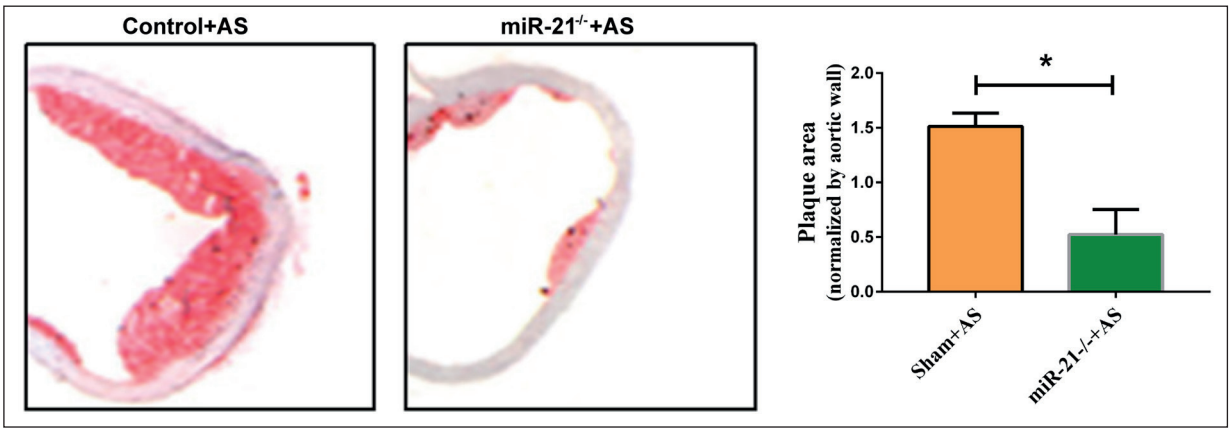

Figure 1. Determination of the level of AS in rats detected by oil red staining and statistical graphs (Magnification: 10×). *$p < 0.05$.

Cell Activity Test Via MTT

As shown in Figure 4, in MTT cell activity assay, the cell activity in the group given with the ERK signaling pathway inhibitor PD98059 and the Akt inhibitor MK-2206 on the basis of miR- 21 was markedly reduced, and the simultaneous administration of the two inhibitors had synergistic effects on the inhibition of the proliferation of VSMCs. The above results suggest that the effects of miR-21 on the proliferation and migration of VSMCs are mediated by the Akt/ERK signaling pathway.

Discussion

AS is a chronic inflammatory disease of the arterial wall caused by endothelial injury and accumulation of subcutaneous lipoproteins[9], and it is the main cause of peripheral vascular diseases[10]. Immune system activation and inflammation are intricately involved in AS, and its pathogenesis involves a multi-stage process of immune and non-immune cells on the vascular wall[11]. The research in the past 30 years has revealed the key signaling and molecular regulatory pathways involved in the occurrence and development of AS plaques[12]. In recent years, the emergence of miRNA, an important regulatory factor in pathophysiological processes, such as cell adhesion[13], proliferation[14], lipid uptake[15], and inflammatory mediators[16], as well as the production of inflammatory mediators, has provided new molecular insights into the effects of miRNAs on these pathways in AS and may be identified as a new therapeutic target[17]. In addition, the detectability of miRNAs

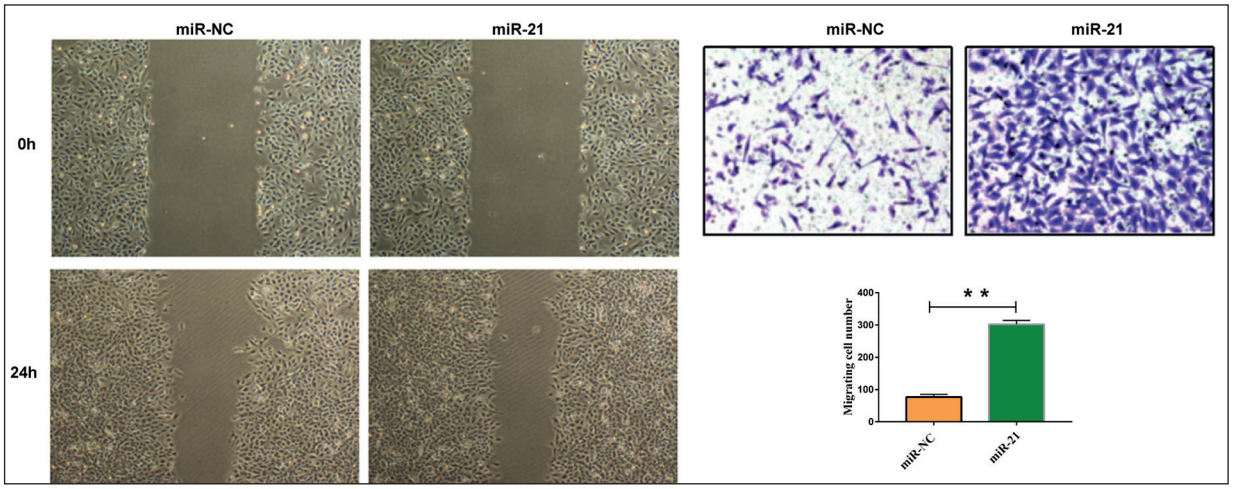

Figure 2. Migration and invasion levels of VSMCs verified *via* wound healing assay (*left*) and transwell assay (*right*) (Magnification: 40×). *$p < 0.001$.

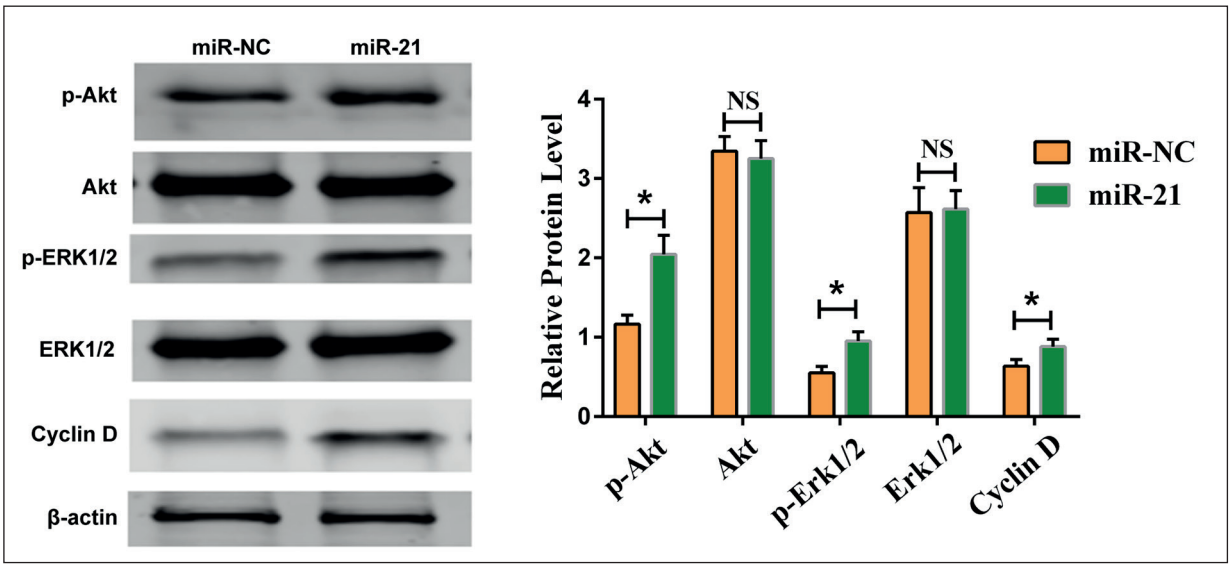

Figure 3. Effects of the miR-21 overexpression on the protein levels of Cyclin D, p-Akt, Akt, p-ERK1/2, and ERK1/2 detected *via* Western blotting. *$p < 0.05$.

in cells and blood circulation improves their potential as biomarkers for diagnosis/prognosis or response to cardiovascular therapy.

As a key mediator in inflammation, studies have shown that neutralizing miR-21 can reduce mechanical balloon injury in response to intimal injury, and miR-21 may promote the proliferation because it can target phosphatases and tensin homologues in VSMCs and indirectly increase anti-apoptosis gene Bcl-2[18]. In regulating VSMC contraction and relaxation phenotypes, the miR-21 expression is significantly increased in dedifferentiated VSMCs compared with that in de-differentiated VSMCs. Consistent with the expression of miR-21 in rodent vascular injury, the delivery of miR-21-resistant coated stents to balloon-injured human mammary arteries also displays a protective effect on the formation of neointimal injury[19]. Investigations have verified that inhibiting miR-21-5p can suppress the proliferation and angiogenesis of human retinal microvascular endothelial cells induced by high glucose through regulating the Akt/ERK pathway[20]. Therefore, further research is still needed to verify whether inhibiting the proliferation-promoting effect of miR-21 can reduce the progression of AS in non-mechanically injured blood vessels and its specific mechanism of action.

Previous researches[18,19] have found that miR-21 is up-regulated in AS and peripheral vascular diseases. In this study, rats fed with a high-fat diet were used to induce the AS model, and the

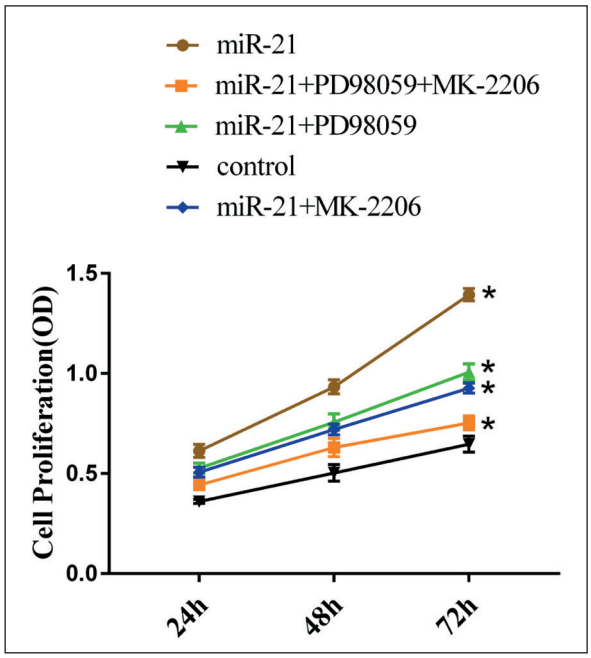

Figure 4. Activity of VSMCs in rats detected via MTT assay. *$p < 0.05$.

situation of thoracic aorta plaques was observed *via* oil red staining. The results showed that there was an evident AS plaque formation on the aorta wall of NC rats, and there was a large amount of lipid infiltration, indicating that the rat model of AS is successfully established. However, aortic plaques in miR-21 knockdown rats were remarkably decreased, and lipid infiltration was reduced, indicating that inhibiting miR-21 can prevent the occurrence and development of AS.

At the cellular level, it was found through wound healing and transwell assays that miR-21 promoted angiogenesis and plaque formation by stimulating the proliferation and migration of vascular fibroblasts in AS, which might aggravate the instability of AS plaques, and the uncontrolled proliferation of smooth muscle cells might lead to vascular stenosis or even blood supply obstruction and thrombosis. In the rescue experiment, it was observed that blocking the Akt/ERK pathway activated by miR-21 is expected to alleviate the hyperproliferative reaction of smooth muscle cells in arterial vascular diseases and maintain plaque stability. It also fully indicates that miR-21 may be a predictor of AS and may become a biochemical indicator for the early identification of AS and peripheral vascular diseases. With the progress of molecular medicine research, miR-21 may become a potential therapeutic target for AS.

Conclusions

We found that miR-21 promotes the proliferation and migration of VSMCs by activating the Akt/ERK pathway and aggravates AS. Knocking down miR-21 or inhibiting the Akt/ERK pathway can suppress the activation of VSMCs.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) de Vries MR, Quax PH. Plaque angiogenesis and its relation to inflammation and atherosclerotic plaque destabilization. Curr Opin Lipidol 2016; 27: 499-506.
2) Li FP, Lin DQ, Gao LY. LncRNA TUG1 promotes proliferation of vascular smooth muscle cell and atherosclerosis through regulating miRNA-21/PTEN axis. Eur Rev Med Pharmacol Sci 2018; 22: 7439-7447.
3) Kolodgie FD, Burke AP, Farb A, Gold HK, Yuan J, Narula J, Finn AV, Virmani R. The thin-cap fibroatheroma: a type of vulnerable plaque: the major precursor lesion to acute coronary syndromes. Curr Opin Cardiol 2001; 16: 285-292.
4) Rzucidlo EM, Martin KA, Powell RJ. Regulation of vascular smooth muscle cell differentiation. J Vasc Surg 2007; 45 Suppl A: A25-A32.
5) Louis SF, Zahradka P. Vascular smooth muscle cell motility: from migration to invasion. Exp Clin Cardiol 2010; 15: e75-e85.
6) Chistiakov DA, Orekhov AN, Bobryshev YV. Vascular smooth muscle cell in atherosclerosis. Acta Physiol (Oxf) 2015; 214: 33-50.
7) Wang Y, Qiu J, Luo S, Xie X, Zheng Y, Zhang K, Ye Z, Liu W, Gregersen H, Wang G. High shear stress induces atherosclerotic vulnerable plaque formation through angiogenesis. Regen Biomater 2016; 3: 257-267.
8) Feinberg MW, Moore KJ. MicroRNA regulation of atherosclerosis. Circ Res 2016; 118: 703-720.
9) Hansson GK, Libby P, Tabas I. Inflammation and plaque vulnerability. J Intern Med 2015; 278: 483-493.
10) Hall A, Smolij C, Moughan B, Kechli A, Aronoff S. Identifying persistent asthma in children: a comparison of healthcare effectiveness data and information set criteria and national heart, lung, and blood institute guidelines. J Healthc Qual 2018; 40: e20-e25.
11) Hansson GK, Hermansson A. The immune system in atherosclerosis. Nat Immunol 2011; 12: 204-212.
12) Weber C, Noels H. Atherosclerosis: current pathogenesis and therapeutic options. Nat Med 2011; 17: 1410-1422.
13) Valastyan S, Weinberg RA. Roles for microRNAs in the regulation of cell adhesion molecules. J Cell Sci 2011; 124: 999-1006.
14) Shenoy A, Blelloch RH. Regulation of microRNA function in somatic stem cell proliferation and differentiation. Nat Rev Mol Cell Biol 2014; 15: 565-576.
15) Vickers KC, Remaley AT. Lipid-based carriers of microRNAs and intercellular communication. Curr Opin Lipidol 2012; 23: 91-97.
16) Mi S, Zhang J, Zhang W, Huang RS. Circulating microRNAs as biomarkers for inflammatory diseases. MicroRNA 2013; 2: 63-71.
17) Ji R, Cheng Y, Yue J, Yang J, Liu X, Chen H, Dean DB, Zhang C. MicroRNA expression signature and antisense-mediated depletion reveal an essential role of MicroRNA in vascular neointimal lesion formation. Circ Res 2007; 100: 1579-1588.
18) Wang D, Deuse T, Stubbendorff M, Chernogubova E, Erben RG, Eken SM, Jin H, Li Y, Busch A, Heeger CH,

Behnisch B, Reichenspurner H, Robbins RC, Spin JM, Tsao PS, Schrepfer S, Maegdefessel L. Local MicroRNA modulation using a novel anti-miR-21-eluting stent effectively prevents experimental in-stent restenosis. Arterioscler Thromb Vasc Biol 2015; 35: 1945-1953.

19) Qiu F, Tong H, Wang Y, Tao J, Wang H, Chen L. Inhibition of miR-21-5p suppresses high glucose-induced proliferation and angiogenesis of human retinal microvascular endothelial cells by the regulation of AKT and ERK pathways via maspin. Biosci Biotechnol Biochem 2018; 82: 1366-1376.

20) Sheedy FJ. Turning 21: induction of miR-21 as a key switch in the inflammatory response. Front Immunol 2015; 6: 19.