Tohoku J. Exp. Med., 2024, **262**, 13-22

OxLDL-Stimulated Macrophages Transmit Exosomal MicroRNA-320b to Aggravate Viability, Invasion, and Phenotype Switching via Regulating PPARGC1A-Mediated MEK/ERK Pathway in Proatherogenic Vascular Smooth Muscle Cells

Lingyun Ren,[1,*] Shanshan Chen[2,*] and Wei Liu[1]

[1]Department of Anesthesiology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China
[2]Key Laboratory for Molecular Diagnosis of Hubei Province, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, Wuhan, Hubei, China

Our previous study revealed oxidized-low density lipoprotein (oxLDL)-stimulated macrophages delivered exosomes to exacerbate vascular smooth muscle cell (VSMC) viability and invasion; and microRNA-320b was enriched in exosomes from oxLDL-stimulated macrophages. This study aimed to further explore molecular mechanisms of exosomal microRNA-320b from oxLDL-stimulated macrophages on cellular functions of VSMCs. Exosomes from oxLDL-stimulated macrophages with microRNA-320b mimic/inhibitor transfection were used to treat VSMCs. Next, microRNA-320b mimic/inhibitor, and microRNA-320b mimic with or without peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) overexpression vector were transfected into VSMCs. Viability, invasion, apoptosis, contractile/synthetic phenotype markers, and MEK/ERK pathway were detected in VSMCs. Exosomes from microRNA-320b mimic-treated macrophages promoted viability, invasion, and synthetic phenotype marker osteopontin, while suppressed apoptosis and contractile phenotype marker α-smooth muscle actin in VSMCs. Importantly, direct microRNA-320b mimic treatment aggravated viability, invasion, and synthetic phenotype transition in VSMCs. However, microRNA-320b inhibitor showed the opposite effects as microRNA-320b mimic. Next, luciferase reporter gene assay showed that microRNA-320b directly bound to PPARGC1A; microRNA-320b also inversely regulated PPARGC1A in VSMCs. Moreover, the effect of microRNA-320b mimic on cellular functions of VSMCs was hampered by PPARGC1A overexpression vector (all $P < 0.05$). Additionally, microRNA-320b activated MEK/ERKT pathway, which was also suppressed by PPARGC1A overexpression vector (all $P < 0.05$). OxLDL-stimulated macrophages deliver exosomal microRNA-320b to exacerbate viability, invasion, and synthetic phenotype transition in VSMCs via modulating PPARGC1A-mediated MEK/ERK pathway, thus participating in the progression of atherosclerosis.

Keywords: atherosclerosis; exosome; macrophage; microRNA-320b; vascular smooth muscle cell
Tohoku J. Exp. Med., 2024 January, **262** (1), 13-22.
doi: 10.1620/tjem.2023.J082

Introduction

Atherosclerosis is the major cause of cerebral and cardiovascular diseases such as ischemic stroke and acute coronary syndrome, the vital reasons for disability and mortality worldwide (Bergmark et al. 2022; Bjorkegren and Lusis 2022; Tsao et al. 2023). When symptom onset, patients with cerebral and cardiovascular diseases can be managed by revascularization including thrombolysis, thrombectomy, percutaneous coronary intervention, and coronary artery

Received August 2, 2023; revised and accepted September 23, 2023; J-STAGE Advance online publication October 5, 2023
*These two authors contributed equally to this work.
Correspondence: Wei Liu, Department of Anesthesiology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, No. 26, Shengli Street, Wuhan, Hubei 430013, China.
e-mail: liuwei8255@163.com
Lingyun Ren, Department of Anesthesiology, The Central Hospital of Wuhan, Tongji Medical College, Huazhong University of Science and Technology, No. 26, Shengli Street, Wuhan, Hubei 430013, China.
e-mail: renpizhi5@163.com

bypass grafting (Association of Cardiovascular Nursing and Allied Professions et al. 2022; Widimsky et al. 2023). Nevertheless, patients may still suffer from neurological or cardiac dysfunction and artery restenosis (Mendelson and Prabhakaran 2021; Rost et al. 2022; Eid et al. 2023). Therefore, it is even more critical to prevent or even reverse the pathogenesis and progression of atherosclerosis to avoid the possible above consequences, which is, unfortunately, still lacking (Bam et al. 2022; Fegers-Wustrow et al. 2022). A potential solution is to explore the key molecules involved in the pathogenesis and progression of atherosclerosis, thus providing therapeutic targets for atherosclerosis.

Macrophages are thought to be critically involved in the pathogenesis of atherosclerosis, which promotes the inflammatory level and engages in the formation and rupture of atherosclerotic plaque (Lin et al. 2021; Eshghjoo et al. 2022). In recent years, studies have also revealed that exosomes secreted by macrophages could deliver their contents, such as microRNAs (miRNAs), proteins, and DNAs, to participate in atherosclerosis (Chen et al. 2022; Liu et al. 2022; Ren et al. 2022). For instance, a previous study suggests that miR-16-5p from macrophage-derived exosomes exacerbates inflammation and oxidative stress in atherosclerosis mice through modulating mothers against decapentaplegic homolog 7 (SMAD7) (Chen et al. 2022). Another research reveals that macrophage-derived exosomes deliver miR-4532 to promote the injury of endothelial cells via targeting the nuclear factor-κB pathway (Liu et al. 2022). Importantly, our previous study discovered that oxidized-low density lipoprotein (oxLDL)-stimulated macrophages deliver exosomes to promote the viability and invasion of proatherogenic vascular smooth muscle cells (VSMCs); notably, our previous work demonstrated that miR-320b was dysregulated in exosomes from oxLDL-stimulated macrophages compared with exosomes from control macrophages, and miR-320b overexpression promoted viability and invasion in proatherogenic VSMCs (Ren et al. 2022). However, the specific molecular mechanisms remain unclear. Therefore, based on our previous study, the current study aimed to evaluate the effect of exosomal miR-320b from oxLDL-stimulated macrophages on the viability, invasion, apoptosis, and phenotype switching in proatherogenic VSMCs, and also to investigate the underlying molecular mechanisms.

Methods

Cell culture

Human VSMC, THP-1, and HEK-293T cells (NCACC, Shanghai, China) were maintained in RPMI-1640 (Servicebio, Wuhan, China) medium plus 10% exosome-free fetal bovine serum (Beyotime, Shanghai, China) and 1% penicillin-streptomycin reagent (Servicebio) at 37°C with 5% CO_2. THP-1 cells were developed into macrophages (THP-1 derived macrophages) after 3 days of incubation with phorbol 12-myristate 13-acetate (PMA) (100 nM; MCE, Shanghai, China) (Ouimet et al. 2017).

OxLDL treatment

The atherosclerosis model *in vitro* was performed according to our previous study (Ren et al. 2022). Briefly, VSMCs and THP-1 derived macrophages were stimulated with oxLDL (100 μg/mL; Beyotime) to mimic the *in vivo* atherosclerosis environment throughout our experiments.

Cell transfection

The miR-320b mimics (miR-mimic), negative control (NC) mimics (NC-mimic), miR-320b inhibitors (miR-inhibitor), and NC inhibitors (NC-inhibitor) were synthesized by Generay (Shanghai, China). For cell transfection, THP-1 derived macrophages or VSMCs were seeded in 6-well dishes (5×10^5 cells/well) and transfected with mimics or inhibitors in the presence of Lipofectamine® 3000 (Invitrogen, Carlsbad, USA). The reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays were completed to assess the expression of miR-320b after being transfected for 48 h.

VSMCs co-cultured with exosomes from THP-1 derived macrophages

The exosomes from THP-1 derived macrophages were isolated after 48 h of transfection according to our previous study (Ren et al. 2022). The expression of miR-320b in the exosomes from THP-1 derived macrophages was detected by RT-qPCR. Then, VSMCs were plated in 6-well dishes (5×10^5 cells/well) and divided into six groups, including the Normal, the Control Exo, the NC-mimic Exo, the miR-mimic Exo, the NC-inhibitor Exo, and the miR-inhibitor Exo groups. Briefly, the VSMCs in the Normal group were not co-cultured with exosomes, and the VSMCs in the other groups were co-cultured with corresponding exosomes (80 μg/mL) of THP-1 derived macrophages. After 48 h of culture, VSMCs were collected to detect cell viability, apoptosis, invasion, and the protein expression of VSMC differentiation markers.

MiR-320b regulation in VSMCs

The transfection of VSMCs was carried out as mentioned above. After 48 h of transfection, the expression of miR-320b and peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PPARGC1A) was determined using RT-qPCR, and the protein level of PGC-1α (encoded by PPARGC1A) was assessed by western blot assay. Then, cell viability, apoptosis, invasion, and the protein expression of VSMC differentiation markers were evaluated.

Luciferase reporter gene assay

The prediction of miR-320b target gene was performed using the starBase (https://starbase.sysu.edu.cn). The PPARGC1A wild type (WT) and mutant type (MUT) reporter vectors were provided by Generay. For the luciferase reporter gene assay, HEK-293T cells were co-transfected with mimics (miR-320b mimics or NC mimics) and reporter vectors (PPARGC1A WT or PPARGC1A MUT).

After being transfected for 48 h, luciferase activity was evaluated with the Dual-Lumi™ Luciferase Assay kit (Beyotime).

PPARGC1A regulation in VSMCs

VSMCs were plated in 6-well dishes (5×10^5 cells/well) and transfected with miR-mimic, NC-mimic, PPARGC1A overexpression vectors (oe-PPARGC1A), and NC vectors (oe-NC) (Generay) alone or in combination using Lipofectamine® 3000. Following 48 h of transfection, the RT-qPCR or western blot assay was carried out to assess the PPARGC1A or PGC-1α expression. VSMCs were harvested for the subsequent assays as previously mentioned.

Detection of cell viability, apoptosis, and invasion

The cell viability of VSMCs was assessed with Cell Counting Kit-8 (Solarbio, Beijing, China) following the kit's protocol. For detection of apoptosis, VSMCs were fixed, permeabilized, and incubated with TUNEL reagent (Sangon, Shanghai, China) and DAPI (Beyotime) following the manufacturer's instructions. The apoptotic cells were counted. For cell invasion assays, VSMCs were plated onto the upper chamber of a Matrigel-coated transwell inserts (BD, Franklin Lakes, NJ, USA) with serum-free medium (1×10^5 cells/well), and the lower chamber was filled with complete medium. After being cultured for 24 h, invasive cells were detected using crystal violet (Beyotime) and analyzed under a microscope (Olympus, Beijing, China). The invasive cells were counted. The pictures were taken with a magnification of 200 ×.

RT-qPCR

RNA was extracted with Trizol (Servicebio) following the kit's protocol. The RT-qPCR assays were performed in the presence of the Reverse Transcription Kit and the PCR Kit (Applied Biosystems, Carlsbad, CA, USA). The relative expression of miR-320b or PPARGC1A was calculated using $2^{-\Delta\Delta Ct}$ method. The following primers were utilized in this study: miR-320b (forward: ACACTCCAGCTGGG AACGGGAGAGTTGG, reverse: TGTCGTGGAGTC GGCAATTC), U6 (forward: GCTTCGGCAGCAC ATATACTAA, reverse: CGAATTTGCGTGTCATCCTT), PPARGC1A (forward: GCTTTCTGGGTGGACTCAAGT, reverse: GAGGGCAATCCGTCTTCATCC), and GAPDH (forward: GAGTCCACTGGCGTCTTCAC, reverse: ATCTTGAGGCTGTTGTCATACTTCT).

Western blot

The proteins of VSMCs were isolated by radioimmunoprecipitation (RIPA) buffer (Beyotime) and quantified via BCA protein assay reagent (Beyotime). Then, proteins were separated with sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transformed to nitrocellulose membrane (PALL, Pensacola, FL, USA), and incubated with specific antibodies (4°C, overnight), including anti-α-smooth muscle actin (α-SMA) (ab124964; 1:1,000), anti-osteopontin (OPN) (ab214050; 1:500), anti-peroxisome proliferator-activated receptor gamma coactivator 1α (PGC-1α) (ab176328; 1:1,000), p-MEK1/2 (ab278723; 1:2,000), MEK1/2 (ab178876; 1:2,000), p-ERK1/2 (ab201015; 1:2,000), ERK1/2 (ab184699; 1:2,000), and anti-GAPDH (ab128915; 1:5,000). The ECL Kit (Beyotime) was adopted to detect the immunoreactive bands after 1 h of incubation with secondary antibody (ab201015) at 37°C. The antibodies were all purchased from Abcam (Shanghai, China).

Statistical analysis

Comparisons were completed with One-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism (V7.0; GraphPad Software, Boston, MA, USA). Statistical significance was set at $P < 0.05$. All experiments were triplicated.

Results

Macrophage exosomal miR-320b exacerbated viability, invasion, and synthetic phenotype switching but suppressed apoptosis in proatherogenic VSMCs

The exosomes from oxLDL-stimulated macrophages after transfection were isolated, then they were used to co-culture with oxLDL-stimulated VSMCs (proatherogenic VSMCs) (Fig. 1A). MiR-320b expression was elevated by miR-mimic ($P < 0.001$) but reduced by miR-inhibitor ($P < 0.05$) in macrophages compared with their corresponding NCs (Fig. 1B), indicating that the transfection was successful. The similar trends of miR-320b expression were also observed in the exosomes from macrophages (both $P < 0.01$) (Fig. 1C). Then, cell viability, invasion, and apoptosis were detected in proatherogenic VSMCs after co-culturing with exosomes from macrophages. It was disclosed that exosomes from control macrophages promoted viability, increased invasion, suppressed apoptosis, and suppressed contractile phenotype marker α-SMA while elevated synthetic phenotype marker OPN in proatherogenic VSMCs compared with normal treatment (all $P < 0.05$) (Fig. 1D-J).

Cell viability was increased by exosomes from miR-mimic treated macrophages versus (vs.) exosomes from NC-mimic treated macrophages ($P < 0.01$) (Fig. 1D). By Transwell and TUNEL assays (Fig. 1E), it was found that exosomes from miR-mimic treated macrophages promoted cell invasion ($P < 0.001$) (Fig. 1F) while reduced cell apoptosis ($P < 0.01$) vs. exosomes from NC-mimic treated macrophages (Fig. 1G). Then, α-SMA and OPN were detected by western blot (Fig. 1H), which showed that exosomes from miR-mimic treated macrophages reduced α-SMA ($P < 0.05$) (Fig. 1I) but elevated OPN ($P < 0.01$) vs. exosomes from NC-mimic treated macrophages (Fig. 1J). Reversely, exosomes from miR-inhibitor treated macrophages (vs. exosomes from NC-inhibitor treated macrophages) had the opposite effects on proatherogenic VSMC viability, inva-

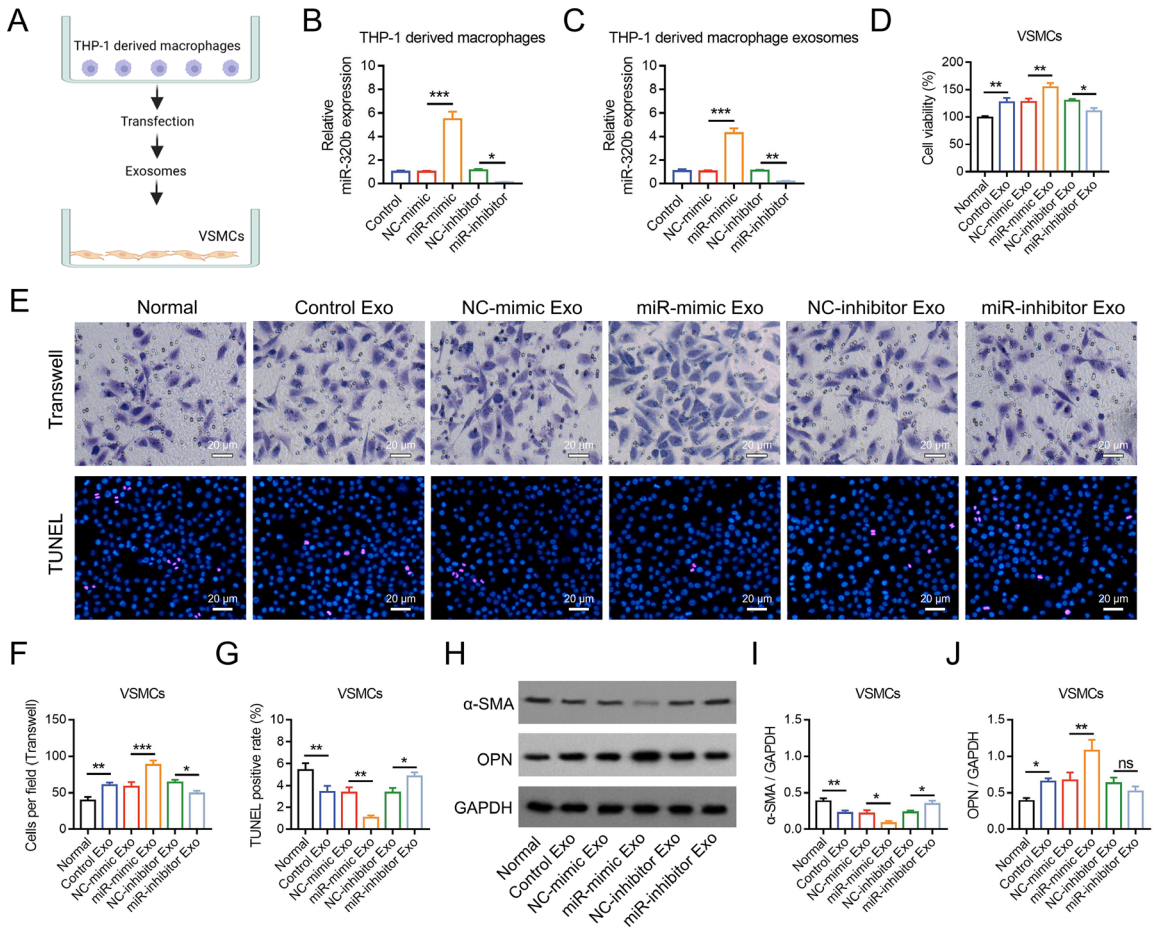

Fig. 1. The effect of exosomal miR-320b from oxLDL-stimulated macrophages on cellular functions of proatherogenic VSMCs.
Illustration of co-culture between exosomes and VSMCs (A). Comparison of miR-320b in macrophages among groups (B). Comparison of miR-320b in exosomes among groups (C). Comparison of VSMC viability among groups (D). Representative images for Transwell and TUNEL assays (E). Comparison of VSMC invasion (F) and apoptosis (G) among groups. Detection of α-SMA and OPN through western blot (H). Comparison of α-SMA (I) and OPN (J) in VSMCs among groups. Cells were treated with 100 μg/mL oxLDL. Data were presented as mean ± SD. n = 3 in each group. Ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

sion, apoptosis, and phenotype markers as exosomes from miR-mimic treated macrophages (all $P < 0.05$ except for OPN) (Fig. 1D-J).

MiR-320b directly aggravated viability, invasion, and synthetic phenotype switching but inhibited apoptosis in proatherogenic VSMCs

MiR-320b was directly modified in proatherogenic VSMCs via miR-mimic or miR-inhibitor, and the transfection efficiencies were validated by RT-qPCR (both $P < 0.01$) (Fig. 2A). It was found that miR-mimic increased viability ($P < 0.001$) (Fig. 2B); it also promoted invasion ($P < 0.01$) but suppressed apoptosis ($P < 0.01$) (Fig. 2C-E) in proatherogenic VSMCs. By western blot (Fig. 2F), it was shown that miR-mimic decreased α-SMA ($P < 0.01$) (Fig. 2G) while elevated OPN ($P < 0.01$) (Fig. 2H) in proatherogenic VSMCs. Moreover, miR-inhibitor showed the opposite effects on viability, invasion, apoptosis, α-SMA, and OPN levels in proatherogenic VSMCs as miR-mimic (all $P < 0.05$ except for OPN) (Fig. 2B-H).

MiR-320b served as a sponge of PPARGC1A

According to the starBase (https://starbase.sysu.edu.cn), PPARGC1A was predicted to be the target of miR-320b. Then, the PPARGC1A MUT was designed according to the binding site between miR-320b and PPARGC1A WT (Fig. 3A). Then, the luciferase reporter gene assay showed that the relative luciferase activity was reduced by miR-320b mimic with the presence of PPARGC1A WT ($P < 0.001$), but unaffected with the presence of PPARGC1A MUT ($P > 0.05$) (Fig. 3B), which suggested the direct binding between miR-320b and PPARGC1A. In proatherogenic VSMCs, miR-mimic suppressed PPARGC1A expression ($P < 0.001$) while miR-inhibitor promoted that ($P < 0.01$) (Fig. 3C); by western blot (Fig. 3D), it was indicated that PGC-1α (the protein encoded by PPARGC1A) also showed

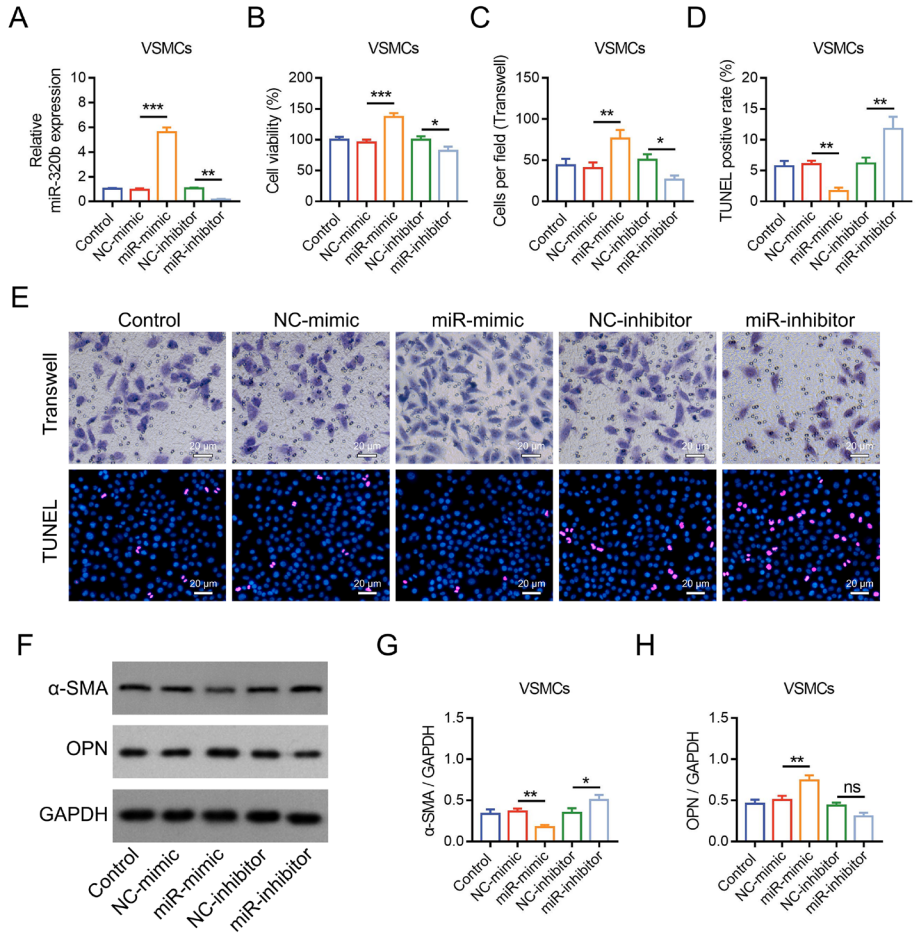

Fig. 2. The effect of miR-320b on cellular functions of proatherogenic VSMCs. Comparison of miR-320b in VSMCs among groups (A). Comparison of VSMC viability (B), invasion (C), and apoptosis (D) among groups. Representative images for Transwell and TUNEL assays (E). Detection of α-SMA and OPN through western blot (F). Comparison of α-SMA (G) and OPN (H) in VSMCs among groups. Cells were treated with 100 μg/mL oxLDL. Data were presented as mean ± SD. n = 3 in each group. Ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

similar trends as PPARGC1A (both $P < 0.05$) (Fig. 3E).

MiR-320b regulated cellular functions in proatherogenic VSMCs via sponging PPARGC1A

Subsequently, PPARGC1A overexpression vector was transfected in proatherogenic VSMCs combined with miR-mimic or NC-mimic. PPARGC1A relative expression was elevated by PPARGC1A overexpression vector ($P < 0.001$); meanwhile, PPARGC1A overexpression vector also hampered the effect of miR-mimic on PPARGC1A relative expression ($P < 0.001$) (Fig. 4A). The expression of PGC-1α showed a similar trend as PPARGC1A relative expression (both $P < 0.01$) (Fig. 4B, C).

PPARGC1A overexpression vector suppressed proatherogenic VSMC viability ($P < 0.01$) and invasion ($P < 0.05$); it also inhibited the effect of miR-mimic on proatherogenic VSMC viability ($P < 0.01$) and invasion ($P < 0.01$) (Fig. 5A, B). Meanwhile, PPARGC1A overexpression vector promoted proatherogenic VSMC apoptosis ($P < 0.01$); it also inhibited the effect of miR-mimic on proatherogenic VSMC apoptosis ($P < 0.01$) (Fig. 5C). The representative images of Transwell and TUNEL assays were shown in Fig. 5D for reference. The detection of α-SMA and OPN (Fig. 5E) suggested that PPPARGC1A overexpression vector promoted the α-SMA expression ($P < 0.01$) and repressed the effect of miR-mimic on α-SMA expression ($P < 0.05$) (Fig. 5F); while OPN showed the opposite trend as α-SMA (both $P < 0.01$) (Fig. 5G).

MiR-mimic activated MEK/ERK pathway by sponging PPARGC1A

Phosphorylation of MEK and ERK was detected by western blot (Fig. 6A). PPARGC1A overexpression vector suppressed the phosphorylation levels of MEK and ERK (both $P < 0.05$). Meanwhile, miR-mimic promoted the phosphorylation levels of MEK and ERK (both $P < 0.05$). However, this promotion by miR-mimic was further hampered by PPARGC1A overexpression vector (both $P < 0.05$) (Fig. 6B, C). These findings suggested that oxLDL-stimulated macrophages deliver exosomal miR-320b to aggravate

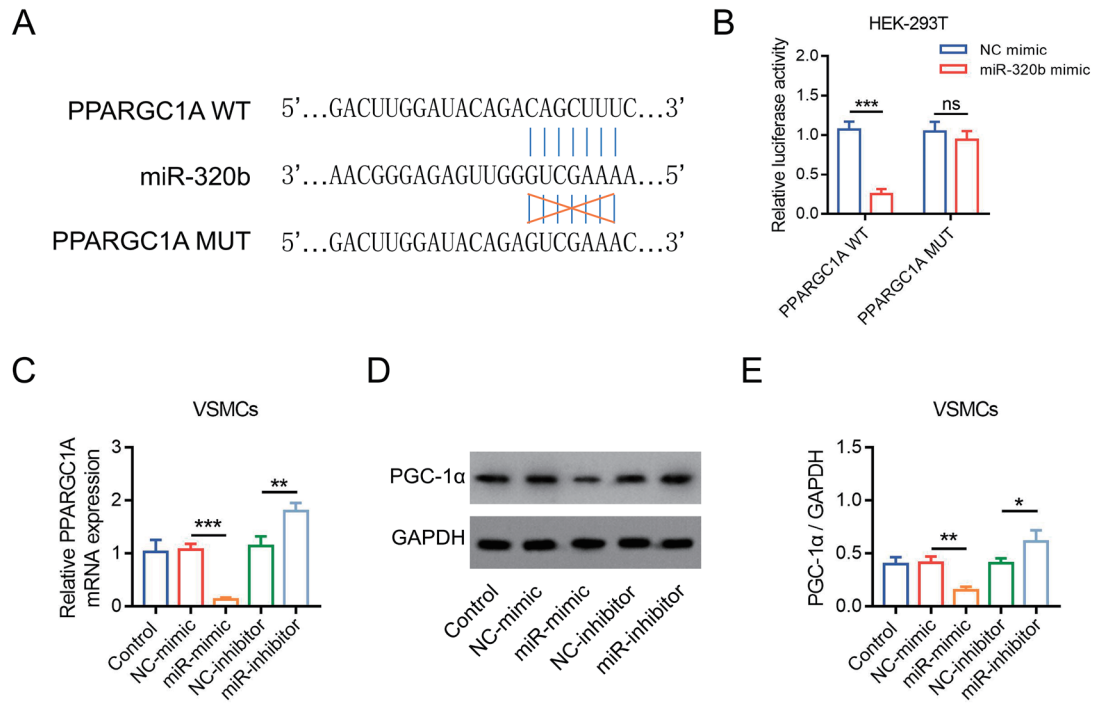

Fig. 3. The binding between miR-320b and PPARGC1A.
Design of PPARGC1A WT and MUT according to the binding site between miR-320b and PPARGC1A (A). Comparison of relative luciferase activity between groups (B). Comparison of PPARGC1A in VSMCs among groups (C). Detection of PGC-1α through western blot (D). Comparison of PGC-1α level in VSMCs among groups (E). Data were presented as mean ± SD. n = 3 in each group. Ns, not significant; *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

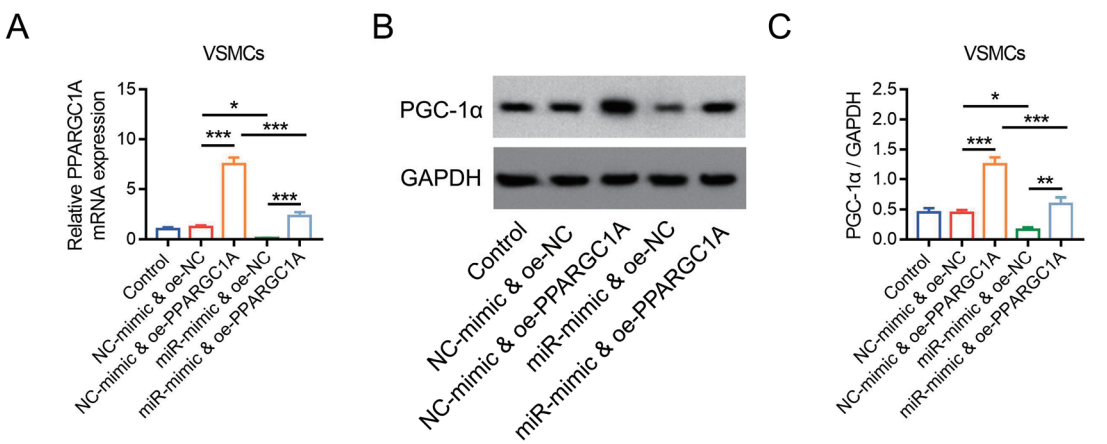

Fig. 4. Transfection efficiency of miR-mimic and oe-PPARGC1A in VSMCs.
Comparison of PPARGC1A in VSMCs among groups (A). Detection of PGC-1α through western blot (B). Comparison of PGC-1α level in VSMCs among groups (C). Cells were treated with 100 μg/mL oxLDL. Data were presented as mean ± SD. n = 3 in each group. *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

the viability, invasion, and synthetic phenotype switching through regulating PPARGC1A-mediated MEK/ERK pathway in proatherogenic VSMCs (Supplementary Fig. S1).

Discussion

MiR-320b is recognized as a critical miRNA involved in atherosclerosis (Huang et al. 2014; Zhang et al. 2016; Barrera-Vazquez et al. 2022; Ren et al. 2022; Wang et al. 2023). From the clinical perspective, it is reported that miR-320b is related to acute ischemic stroke independent of geographical location and tissue (Barrera-Vazquez et al. 2022). Another study discloses that miR-320b is dysregulated in subjects with carotid atherosclerotic plaque compared with healthy subjects (Zhang et al. 2016). Moreover, the dysregulation of circulating miR-320b is associated with the occurrence of acute myocardial infarction (Huang

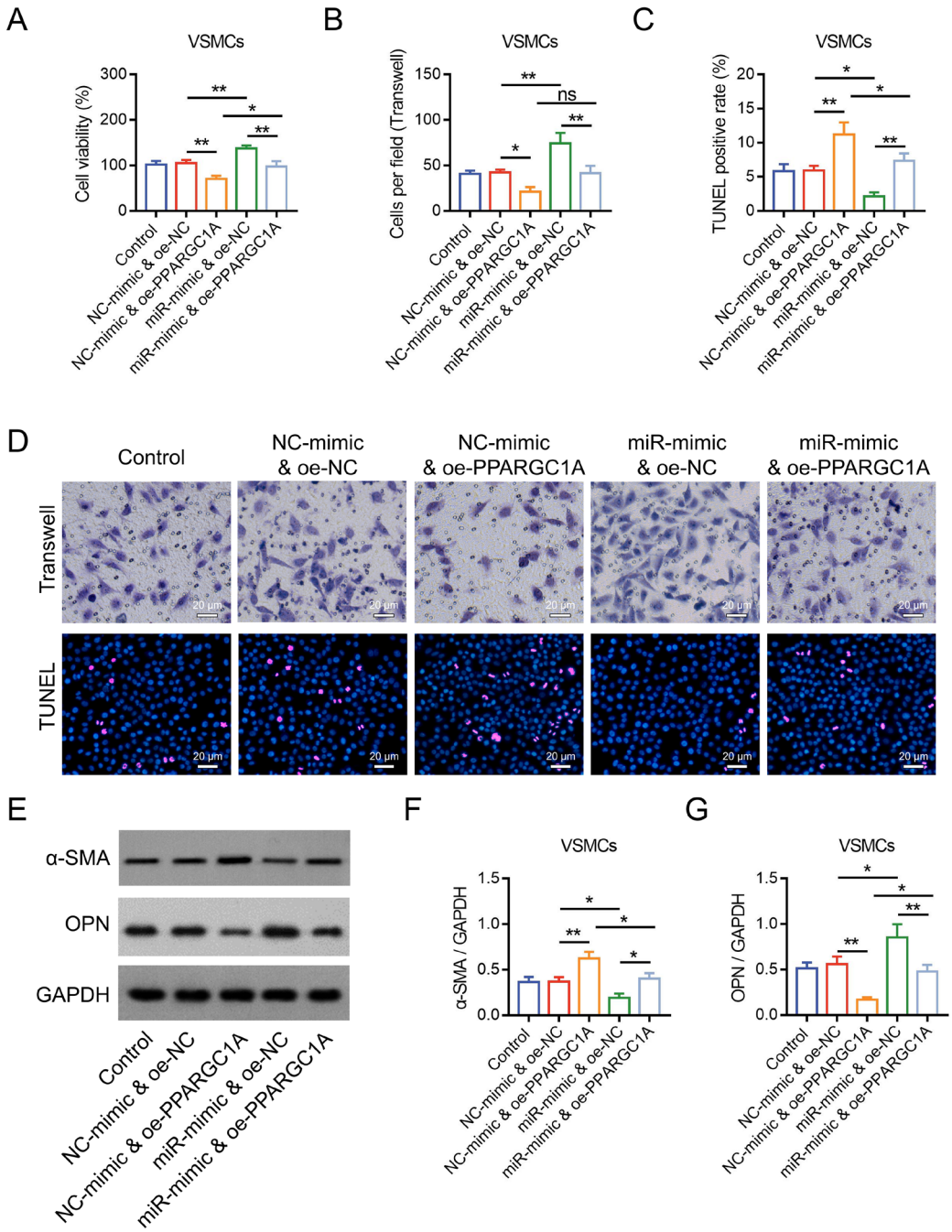

Fig. 5. The effect of PPARGC1A on miR-320b-mediated cellular functions of proatherogenic VSMCs. Comparison of VSMC viability (A), invasion (B), and apoptosis (C) among groups. Representative images for Transwell and TUNEL assays (D). Detection of α-SMA and OPN through western blot (E). Comparison of α-SMA (F) and OPN (G) in VSMCs among groups. Cells were treated with 100 μg/mL oxLDL. Data were presented as mean ± SD. n = 3 in each group. Ns, not significant; *$P < 0.05$; **$P < 0.01$.

et al. 2014). Apart from these clinical studies, it is also reported that miR-320b suppresses high-density lipoprotein- and apolipoprotein A1-mediated cholesterol efflux from macrophages; its administration *in vivo* also represses cholesterol efflux from peritoneal macrophages and increases the accumulation of low-density lipoprotein-cholesterol, thus further aggravating atherosclerotic plaque size and inflammatory level (Wang et al. 2023). Meanwhile, our previous study discloses that miR-320b was dysregulated in exosomes from oxLDL-stimulated macrophages, and exosomes from oxLDL-stimulated macrophages exacerbated viability and invasion in proatherogenic VSMCs (Ren et al. 2022). However, it is unclear whether exosomal miR-320b from oxLDL-stimulated macrophages could regulate the cellular functions of proatherogenic VSMCs. In the current study, several interesting findings were disclosed. First,

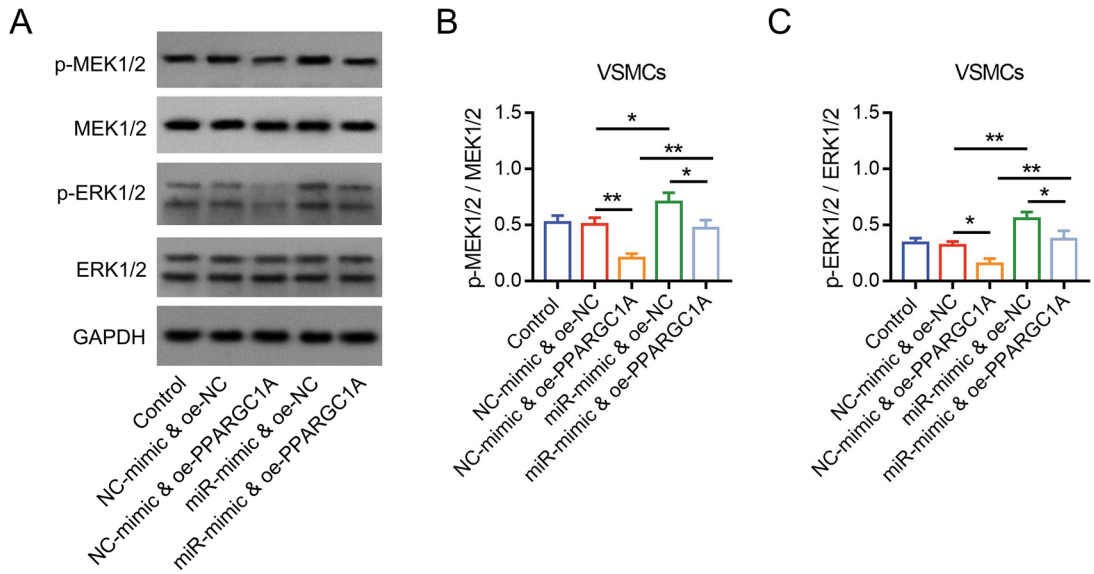

Fig. 6. The effect of miR-320b and PPARGC1A on MEK/ERK pathway.
Detection of p-MEK1/2 and p-ERK1/2 through western blot (A). Comparison of p-MEK1/2 (B) and p-ERK1/2 (C) in VSMCs among groups. Cells were treated with 100 μg/mL oxLDL. Data were presented as mean ± SD. n = 3 in each group. *$P < 0.05$; **$P < 0.01$.

exosomes from oxLDL-stimulated macrophages promoted viability, invasion, and synthetic phenotype switching while suppressed apoptosis in proatherogenic VSMCs, which was in line with our previous research (Ren et al. 2022). Second, exosomal miR-320b from oxLDL-stimulated macrophages aggravated viability, invasion, and synthetic phenotype switching while suppressed apoptosis in proatherogenic VSMCs, indicating a stronger ability of proliferation and migration than the contractile phenotype and thus contributing to the pathogenesis of atherosclerosis (Durham et al. 2018). Possible explanations were that; (1) miR-320b might activate the downstream MEK/ERK signaling, which could directly regulate these cellular functions in proatherogenic VSMCs; and (2) miR-320b might serve as a sponge to suppress its target, such as PPARGC1A, a critical regulator of atherosclerosis, thus further exacerbating the cellular functions of proatherogenic VSMCs (Wang et al. 2023).

In order to further explore the molecular mechanisms of miR-320b exacerbating the cellular functions of proatherogenic VSMCs, the current study identified PPARGC1A as the potential target of miR-320b. Meanwhile, the direct binding between PPARGC1A and miR-320b was confirmed by luciferase reporter gene assay. PGC-1α interacts with various transcription factors and participates in multiple biological activities of cells (Maciejewska-Skrendo et al. 2022). In terms of its implication in atherosclerosis, several studies have reported that the polymorphisms of PPARGC1A are associated with coronary artery syndrome (Zhang et al. 2008; Maciejewska-Skrendo et al. 2019; Schillemans et al. 2022). Meanwhile, it is also reported that the overexpression of PPARGC1A reduces the size of atherosclerotic plaque by 40% in apolipoprotein E-knockout mice (Shimba et al. 2019). Another previous study suggests that PPARGC1A is detected in macrophages of human atherosclerotic lesions and is inversely associated with atherosclerosis progression; its deletion in macrophages accelerates the formation of atherosclerosis in mice (McCarthy et al. 2013). In the current study, it was disclosed that PPARGC1A overexpression inhibited viability, invasion, and synthetic phenotype switching while promoted apoptosis in proatherogenic VSMCs. Possible explanations might be that; (1) PPARGC1A could modulate lipid and energy metabolism to regulate these cellular functions in proatherogenic VSMCs (Zhu et al. 2009; Zhang et al. 2020); (2) PPARGC1A might regulate the downstream MEK/ERK signaling pathway to exert these effects on proatherogenic VSMCs (Pintus et al. 2003; Huang et al. 2020). More importantly, it was shown that miR-320b regulated the cellular functions of proatherogenic VSMCs through sponging PPARGC1A.

The MEK/ERK signaling is a fundamental pathway that regulates various cellular functions, including cell growth, cell survival, migration and invasion, T cell differentiation, etc. (Ullah et al. 2022). Meanwhile, the MEK/ERK pathway is also considered to be closely involved in the pathogenesis and progression of atherosclerosis (Pintus et al. 2003; Peng et al. 2022; Zhang et al. 2022). Since previous studies have indicated that the MEK/ERK pathway is one of the downstream signalings of PPARGC1A (Huang et al. 2020; Nam et al. 2020), the current study further explored the effect of miR-320b and PPARC1A in proatherogenic VSMCs. It was found that miR-320b activated the MEK/ERK pathway through sponging PPARGC1A. These findings could also explain the promotion of viability, inva-

sion, and synthetic phenotype switching while suppression of apoptosis in proatherogenic VSMCs by miR-320b.

Collectively, oxLDL-stimulated macrophages deliver exosomal miR-320b to aggravate the viability, invasion, and synthetic phenotype switching through regulating PPARGC1A-mediated MEK/ERK pathway in proatherogenic VSMCs. These findings suggest that exosomal miR-320b from macrophages may be a potential therapeutic target for atherosclerosis. However, further validation is needed.

Acknowledgments

This study was supported by Wuhan Municipal Health Commission (No. WX21A09) and Wuhan Knowledge Innovation Special Project (No. 2022020801010537).

Conflict of Interest

The authors declare no conflict of interest.

References

Association of Cardiovascular Nursing and Allied Professions (ACNAP); Association for Acute CardioVascular Care (ACVC); European Association of Percutaneous Cardiovascular Interventions (EAPCI); EURObservational Research Programme (EORP); ESC Patient Forum; ESC Working Group on Thrombosis and ESC Committee for Young Cardiovascular Professionals, Batra, G., Aktaa, S., Wallentin, L., Maggioni, A.P., Ludman, P., Erlinge, D., Casadei, B. & Gale, C.P. (2022) Data standards for acute coronary syndrome and percutaneous coronary intervention: the European Unified Registries for Heart Care Evaluation and Randomised Trials (EuroHeart). *Eur. Heart J.*, **43**, 2269-2285.

Bam, K., Olaiya, M.T., Cadilhac, D.A., Donnan, G.A., Murphy, L. & Kilkenny, M.F. (2022) Enhancing primary stroke prevention: a combination approach. *Lancet Public Health*, **7**, e721-e724.

Barrera-Vazquez, O.S., Gomez-Verjan, J.C., Ramirez-Aldana, R., Torre, P.G. & Rivero-Segura, N.A. (2022) Structural and pharmacological network analysis of miRNAs involved in acute ischemic stroke: a systematic review. *Int. J. Mol. Sci.*, **23**, 4663.

Bergmark, B.A., Mathenge, N., Merlini, P.A., Lawrence-Wright, M.B. & Giugliano, R.P. (2022) Acute coronary syndromes. *Lancet*, **399**, 1347-1358.

Bjorkegren, J.L.M. & Lusis, A.J. (2022) Atherosclerosis: recent developments. *Cell*, **185**, 1630-1645.

Chen, F., Li, J., She, J., Chen, T. & Yuan, Z. (2022) Exosomal microRNA-16-5p from macrophage exacerbates atherosclerosis via modulating mothers against decapentaplegic homolog 7. *Microvasc. Res.*, **142**, 104368.

Durham, A.L., Speer, M.Y., Scatena, M., Giachelli, C.M. & Shanahan, C.M. (2018) Role of smooth muscle cells in vascular calcification: implications in atherosclerosis and arterial stiffness. *Cardiovasc. Res.*, **114**, 590-600.

Eid, M.M., Mostafa, M.R., Alabdouh, A., AbdelQadir, Y.H., Mohamed, S., Abdelazeem, B., Ismayl, M., Abusnina, W., Alweis, R., Corteville, D.C.M., Balmer-Swain, M., Paul, T.K. & Mamas, M.A. (2023) Long-term outcomes of acute myocardial infarction in pre-existing coronary artery ectasia: a systematic review and meta-analysis. *Curr. Probl. Cardiol.*, **48**, 101626.

Eshghjoo, S., Kim, D.M., Jayaraman, A., Sun, Y. & Alaniz, R.C. (2022) Macrophage polarization in atherosclerosis. *Genes* (*Basel*), **13**, 756.

Fegers-Wustrow, I., Gianos, E., Halle, M. & Yang, E. (2022) Comparison of American and European guidelines for primary prevention of cardiovascular disease: JACC guideline comparison. *J. Am. Coll. Cardiol.*, **79**, 1304-1313.

Huang, J.B., Hsu, S.P., Pan, H.Y., Chen, S.D., Chen, S.F., Lin, T.K., Liu, X.P., Li, J.H., Chen, N.C., Liou, C.W., Hsu, C.Y., Chuang, H.Y. & Chuang, Y.C. (2020) Peroxisome proliferator-activated receptor gamma coactivator 1alpha activates vascular endothelial growth factor that protects against neuronal cell death following status epilepticus through PI3K/AKT and MEK/ERK signaling. *Int. J. Mol. Sci.*, **21**, 7247.

Huang, S., Chen, M., Li, L., He, M., Hu, D., Zhang, X., Li, J., Tanguay, R.M., Feng, J., Cheng, L., Zeng, H., Dai, X., Deng, Q., Hu, F.B. & Wu, T. (2014) Circulating MicroRNAs and the occurrence of acute myocardial infarction in Chinese populations. *Circ. Cardiovasc. Genet.*, **7**, 189-198.

Lin, P., Ji, H.H., Li, Y.J. & Guo, S.D. (2021) Macrophage plasticity and atherosclerosis therapy. *Front. Mol. Biosci.*, **8**, 679797.

Liu, P., Wang, S., Wang, G., Zhao, M., Du, F., Li, K., Wang, L., Wu, H., Chen, J., Yang, Y. & Su, G. (2022) Macrophage-derived exosomal miR-4532 promotes endothelial cells injury by targeting SP1 and NF-kappaB P65 signalling activation. *J. Cell. Mol. Med.*, **26**, 5165-5180.

Maciejewska-Skrendo, A., Massidda, M., Tocco, F. & Leznicka, K. (2022) The influence of the differentiation of genes encoding peroxisome proliferator-activated receptors and their coactivators on nutrient and energy metabolism. *Nutrients*, **14**, 5378.

Maciejewska-Skrendo, A., Pawlik, A., Sawczuk, M., Rac, M., Kusak, A., Safranow, K. & Dziedziejko, V. (2019) PPARA, PPARD and PPARG gene polymorphisms in patients with unstable angina. *Gene*, **711**, 143947.

McCarthy, C., Lieggi, N.T., Barry, D., Mooney, D., de Gaetano, M., James, W.G., McClelland, S., Barry, M.C., Escoubet-Lozach, L., Li, A.C., Glass, C.K., Fitzgerald, D.J. & Belton, O. (2013) Macrophage PPAR gamma Co-activator-1 alpha participates in repressing foam cell formation and atherosclerosis in response to conjugated linoleic acid. *EMBO Mol. Med.*, **5**, 1443-1457.

Mendelson, S.J. & Prabhakaran, S. (2021) Diagnosis and management of transient ischemic attack and acute ischemic stroke: a review. *JAMA*, **325**, 1088-1098.

Nam, H., Kundu, A., Brinkley, G.J., Chandrashekar, D.S., Kirkman, R.L., Chakravarthi, B., Orlandella, R.M., Norian, L.A., Sonpavde, G., Ghatalia, P., Fei, F., Wei, S., Varambally, S. & Sudarshan, S. (2020) PGC1alpha suppresses kidney cancer progression by inhibiting collagen-induced SNAIL expression. *Matrix Biol.*, **89**, 43-58.

Ouimet, M., Ediriweera, H., Afonso, M.S., Ramkhelawon, B., Singaravelu, R., Liao, X., Bandler, R.C., Rahman, K., Fisher, E.A., Rayner, K.J., Pezacki, J.P., Tabas, I. & Moore, K.J. (2017) microRNA-33 regulates macrophage autophagy in atherosclerosis. *Arterioscler. Thromb. Vasc. Biol.*, **37**, 1058-1067.

Peng, Q., Yin, R., Zhu, X., Jin, L., Wang, J., Pan, X. & Ma, A. (2022) miR-155 activates the NLRP3 inflammasome by regulating the MEK/ERK/NF-kappaB pathway in carotid atherosclerotic plaques in ApoE(-/-) mice. *J. Physiol. Biochem.*, **78**, 365-375.

Pintus, G., Tadolini, B., Posadino, A.M., Sanna, B., Debidda, M., Carru, C., Deiana, L. & Ventura, C. (2003) PKC/Raf/MEK/ERK signaling pathway modulates native-LDL-induced E2F-1 gene expression and endothelial cell proliferation. *Cardiovasc. Res.*, **59**, 934-944.

Ren, L., Chen, S., Yao, D. & Yan, H. (2022) OxLDL-stimulated macrophage exosomes promote proatherogenic vascular smooth muscle cell viability and invasion via delivering miR-186-5p then inactivating SHIP2 mediated PI3K/AKT/mTOR pathway. *Mol. Immunol.*, **146**, 27-37.

Rost, N.S., Brodtmann, A., Pase, M.P., van Veluw, S.J., Biffi, A., Duering, M., Hinman, J.D. & Dichgans, M. (2022) Post-stroke cognitive impairment and dementia. *Circ. Res.*, **130**, 1252-1271.

Schillemans, T., Tragante, V., Maitusong, B., Gigante, B., Cresci, S., Laguzzi, F., Vikstrom, M., Richards, M., Pilbrow, A., Cameron, V., Foco, L., Doughty, R.N., Kuukasjarvi, P., Allayee, H., Hartiala, J.A., et al. (2022) Associations of polymorphisms in the peroxisome proliferator-activated receptor gamma coactivator-1 alpha gene with subsequent coronary heart disease: an individual-level meta-analysis. *Front. Physiol.*, **13**, 909870.

Shimba, Y., Togawa, H., Senoo, N., Ikeda, M., Miyoshi, N., Morita, A. & Miura, S. (2019) Skeletal muscle-specific PGC-1alpha overexpression suppresses atherosclerosis in apolipoprotein E-knockout mice. *Sci. Rep.*, **9**, 4077.

Tsao, C.W., Aday, A.W., Almarzooq, Z.I., Anderson, C.A.M., Arora, P., Avery, C.L., Baker-Smith, C.M., Beaton, A.Z., Boehme, A.K., Buxton, A.E., Commodore-Mensah, Y., Elkind, M.S.V., Evenson, K.R., Eze-Nliam, C., Fugar, S., et al. (2023) Heart disease and stroke statistics-2023 update: a report from the American Heart Association. *Circulation*, **147**, e93-e621.

Ullah, R., Yin, Q., Snell, A.H. & Wan, L. (2022) RAF-MEK-ERK pathway in cancer evolution and treatment. *Semin. Cancer Biol.*, **85**, 123-154.

Wang, G., Luo, Y., Gao, X., Liang, Y., Yang, F., Wu, J., Fang, D. & Luo, M. (2023) MicroRNA regulation of phenotypic transformations in vascular smooth muscle: relevance to vascular remodeling. *Cell. Mol. Life Sci.*, **80**, 144.

Widimsky, P., Snyder, K., Sulzenko, J., Hopkins, L.N. & Stetkarova, I. (2023) Acute ischaemic stroke: recent advances in reperfusion treatment. *Eur. Heart J.*, **44**, 1205-1215.

Zhang, J., Zhao, W.R., Shi, W.T., Tan, J.J., Zhang, K.Y., Tang, J.Y., Chen, X.L. & Zhou, Z.Y. (2022) Tribulus terrestris L. extract ameliorates atherosclerosis by inhibition of vascular smooth muscle cell proliferation in ApoE(-/-) mice and A7r5 cells via suppression of Akt/MEK/ERK signaling. *J. Ethnopharmacol.*, **297**, 115547.

Zhang, Q., Ma, X.F., Dong, M.Z., Tan, J., Zhang, J., Zhuang, L.K., Liu, S.S. & Xin, Y.N. (2020) MiR-30b-5p regulates the lipid metabolism by targeting PPARGC1A in Huh-7 cell line. *Lipids Health Dis.*, **19**, 76.

Zhang, R., Qin, Y., Zhu, G., Li, Y. & Xue, J. (2016) Low serum miR-320b expression as a novel indicator of carotid atherosclerosis. *J. Clin. Neurosci.*, **33**, 252-258.

Zhang, Y., Xu, W., Li, X., Tang, Y., Xie, P., Ji, Y., Fan, L. & Chen, Q. (2008) Association between PPARGC1A gene polymorphisms and coronary artery disease in a Chinese population. *Clin. Exp. Pharmacol. Physiol.*, **35**, 1172-1177.

Zhu, L., Sun, G., Zhang, H., Zhang, Y., Chen, X., Jiang, X., Jiang, X., Krauss, S., Zhang, J., Xiang, Y. & Zhang, C.Y. (2009) PGC-1alpha is a key regulator of glucose-induced proliferation and migration in vascular smooth muscle cells. *PLoS One*, **4**, e4182.

Supplementary Files

Please find supplementary file(s);
https://doi.org/10.1620/tjem.2023.J082