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Exosome-mediated miR-106a-3p derived from ox-LDL exposed macrophages accelerated cell proliferation and repressed cell apoptosis of human vascular smooth muscle cells

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Abstract. – OBJECTIVE: Atherosclerosis (AS) is a leading disease with high mortality and morbidity in the world. It has been demonstrated that exosomes can transfer some miRNAs or proteins to regulate the biological functions of human vascular smooth muscle cells (VSMCs) and promote the progression of AS. In this study, we mainly aimed at exploring potential functions of exosomes derived from ox-LDL exposed macrophages and investigating the potential mechanisms of exosome-mediated miR-106a-3p in regulating VSMCs and promoting AS.

MATERIALS AND METHODS: Ox-LDL was used to treat THP-1 macrophages, CCK-8 assay was performed to detect cell viability, and flow cytometric analysis was used to detect cell apoptosis. Exosomes were isolated and collected with centrifugation, and were determined by transmission electron microscopy and WB assay. RT-PCR was used to detect the expressions of miRNAs in exosomes and VSMCs, WB assay was used to detect protein expressions. MiR-106a-3p mimic was transfected into VSMCs to verify its functions and the Luciferase gene reporter assay was performed to prove the binding site of miR-106a-3p and CASP9. Finally, GW4869, an inhibitor for exosome secretion, was used to block exosome secretion by ox-LDL induced THP-1 and to confirm the effects of miR-106a-3p on cell proliferation and apoptosis in VSMCs.

RESULTS: We found that ox-LDL induced THP-1 could promote cell proliferation and repress cell apoptosis of VSMCs, then, exosomes were successfully isolated, which could promote cell proliferation and repressed cell apoptosis of VSMCs after adding into VSMCs. Furthermore, we found that miR-106a-3p was significantly increased in exosomes from ox-LDL induced THP-1 and its expression was also increased in VSMCs after adding into VSMCs. Moreover, miR-106a-3p overexpression could promote cell viability and repress cell apoptosis, as well as regulate associated protein expressions. Additionally, the Luciferase gene reporter assay confirmed that miR-106a-3p could directly bind with CASP9 and regulate Caspase signaling in VSMCs. Finally, blocking exosomes from ox-LDL induced THP-1 reduced the cell viability and promoted cell apoptosis in VSMCs.

CONCLUSIONS: Above all, this study demonstrated that miR-106a-3p was increased in exosomes from ox-LDL induced THP-1 and it could promote cell proliferation and repress cell apoptosis of VSMCs. We found that the exosomes-mediated miR-106a-3p could directly bind with CASP9 and repress Caspase signaling pathway in VSMCs, which might provide a potential target for treating AS.

Key Words:

Exosomes, MiR-106a-3p, Proliferation, Vascular smooth muscle cells, Macrophages, Atherosclerosis.

Introduction

Atherosclerosis (AS) is a kind of chronic progressive inflammatory disease mainly responsible for coronary heart disease (CHD) and a leading cause of human death worldwide[1-4]. It has been demonstrated that macrophages and vascular smooth muscle cells (VSMCs) are critical for the development of AS[5-7]. Endothelial cells (ECs) are exposed to ox-LDL, which has been revealed to be widely associated with pathogenesis of AS and induce the vascular endothelial injury[8-11]. The activation of VSMCs and macrophages contribute to promote atherosclerotic plaque formation and development of AS[5-7]. However, detailed formation process and interaction between VSMCs and macrophages remain not fully understood; therefore, further studies are needed for a better understanding of AS.

Exosomes are kinds of membranous extracellular vesicles (EVs) that are produced in the endosomal compartment of cells, which range from 30 to

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150 nanometres (nm) in diameter[12-14]. It is unclear whether exosomes have some characters or functions that are from other EVs[14]. It has been revealed that exosomes carry miRNAs, mRNAs, and proteins, which can be transferred into target cells and play some important roles in cancers and AS[15-18]. Li et al[15] reported that the exosomes derived from mesenchymal stem cells could attenuate the progression of AS in ApoE(-/-) mice *via* miR-let7 mediated infiltration and polarization of macrophages. Zhang et al[18] found that exosomes derived from ox-LDL-stimulated macrophages transferred miR-146a and induced neutrophil extracellular traps to promote the progression of AS, but the effects of exosomes between macrophages and VSMCs are not fully understood.

MiRNAs are small non-coding RNAs that can regulate some biological functions by binding to the 3′-untranslated region (3′-UTR) of target genes for miRNAs, and then, inhibiting mRNA translation, thereby affecting the progression in diseases and AS[19-21]. MiR-106a-3p belongs to one of poorly conserved miRNA family members, MiR-106a, which has been revealed to promote cell proliferation and induced Apatinib resistance in some cancers[22-24]. However, whether miR-106a-3p plays some roles in the development of AS is unclear.

In this study, we mainly aimed at exploring the potential functions of exosomes derived from ox-LDL exposed macrophages. We found that miR-106a-3p was increased in exosomes derived from ox-LDL exposed macrophages, then, we aimed at investigating the potential mechanisms.

Materials and Methods

Cell Culture

Human vascular smooth muscle cell line VSMC and human macrophages THP-1 were purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA). Cells were cultured in DMEM/F12 medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum (FBS; Invitrogen, Carlsbad, CA, USA), which was centrifuged at 200,000 g for 18 h to deplete exosomes. The antibiotics penicillin (100 U/ml) and streptomycin (100 μg/mL) were added into the medium, cells were cultured in an incubator with 5% CO_2 at 37°C. VSMCs were co-cultured with THP-1 or ox-LDL induced THP-1, and with an equal volume of complete culture media. The transwell inserts (Costar, NY, USA) were used to co-culture VSMCs and THP-1. The VSMCs were seeded in the lower chambers, and THP-1 or ox-LDL induced THP-1 were seeded in the upper chambers to imitate a non-contacted co-culturing system to simulate VSMCs interactions, which was similar to that in the microenvironment of AS. The pore diameter of upper chambers was 0.4 μm, which was smaller than THP-1 to prevent THP-1 from crossing the membranes; however, it could allow exosomes to pass through.

Exosome Isolation

Exosomes were extracted by using Exosome Precipitation Solution (Exo-Quick; System Bioscience, Mountain View, CA, USA) according to the manufacturer's instructions with some modifications. Appropriate volume of the solution was added into the THP-1-conditioned media and refrigerated at 4°C overnight. After that, the samples were centrifuged at 180 g for 30 mins at 4°C, and then, centrifuged at 700 g for 5 mins. Finally, exosome pellets were resuspended with PBS and stored at -80°C for further studies. Exosomes were resuspended in 500 μl of appropriate cell media and incubated 48 h with recipient cells, and exosome-depleted medium was obtained by ultracentrifugation at 16,500 g for 90 mins.

Transmission Electron Microscopy and Zeta Analysis

The isolated exosomes were identified by transmission electron microscopy (TEM) morphology assessments, 3 μl isolated exosome pellet was placed on formvar carbon-coated 200 mesh copper electron microscopy grids, which was then incubated for 5 mins at room temperature and subjected to standard uranyl acetate staining. The grids were washed with PBS before the observation by TEM (JEOL JEM 1230, Peabody, MA, USA). Micrographs were used to quantify the diameters of the exosomes. We measured the sizes of exosomes by nanoparticle tracking analysis (NTA) with ZetaView PMX 110 (Particle Metrix, Meerbusch, Germany), which was calibrated using 100 nm polystyrene particles.

Cell Transfection

VSMCs were seeded in 6-well plates (1×10^6 cells/well) for 24 h until 50%, then, the culture medium was exchanged and washed with PBS for three times. MiR-106a-3p mimic or miR-NC was respectively transfected into the prepared VSMCs with the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) for 24 h according to the manufacturer's instructions and the cells were harvested for further study.

Treatment of VSMCs with Exosomes or GW4869

VSMCs were seeded in 6-well plates (1×10^6 cells/well) and treated with culture media containing 20 μg exosomes isolated from non-ox-LDL treated THP-1 or ox-LDL induced THP-1, or exosome-free media. The supernatants were harvested after 48 h for further study. For inhibition of exosome secretion assay, GW4869 (Sigma-Aldrich, St. Louis, MO, USA) was used and was dissolved in dimethyl sulfoxide (DMSO; Thermo Fisher Scientific, Waltham, MA, USA) into a stock solution. VSMCs were pre-treated with media containing 20 μM GW4869 for 2 h prior to the treatment with ox-LDL, then, the supernatants were collected after 48 h for further study.

CCK-8 Assay

A Cell Counting Kit-8 (CCK-8) assay (Dojindo, Kumamoto, Japan) was used to detect cell viability of human VSMCs. VSMCs were seeded on 96-well plates (2×10^3 cells/ml) and cultured in an incubator with 5% CO_2 at 37°C. 10 μl CCK-8 agent was treated into the medium after 72 h, which were cultured at darkness for 2 h at 37°C, the viability of VSMCs was measured by CCK-8 assay according to its protocol. Finally, absorbance (OD) was measured at 450 nm by using a microplate reader (Thermo Fisher Scientific, Waltham, MA, USA). Each experiment was repeated for three times and the mean values were calculated.

Flow Cytometric Analysis

Cell apoptotic rates were measured by flow cytometric analysis. The prepared cells were washed for three times and were digested by 0.25% trypsin. The cell precipitations were collected after centrifugation and were added with 75% ethanol at 4°C for 4 h. Then, the cell pellets were stained with FITC-Annexin V (BD Biosciences, Franklin Lakes, NJ, USA) and Propidium iodide (PI; BD Biosciences, Franklin Lakes, NJ, USA) at room temperature for 10 mins. Finally, cell pellets were subjected to a flow cytometry (BD Biosciences, Franklin Lakes, NJ, USA) to analyze cell apoptotic rates. Each experiment was repeated for three times and the mean values were calculated.

RNA Extraction and Quantitative RT-PCR

Total RNAs from VSMCs were extracted by using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to its protocol. Subsequently, mRNA was reverse transcribed into cDNA by using a PrimeScript™ RT reagent Kit (TaKaRa, Otsu, Shiga, Japan) according to its protocol. The qPCR amplification was performed using a TaKaRa system and SYBR Premix Ex Taq II (TaKaRa, Otsu, Shiga, Japan) was used to detect mRNA expressions. The primers for RT-PCR were synthesized by TaKaRa Biotechnology Ltd. (Dalian, Liaoning, China) and were listed in Table I. The relative mRNA expressions were normalized to GAPDH or U6 and $2^{-\Delta\Delta CT}$ method was used to calculate the relative gene expressions.

Protein Extraction and Western Blot Analysis

Total protein was extracted by using a RIPA protein extraction buffer (Beyotime Biotechnology Co., Shanghai, China) with a protease inhibitor (Beyotime Biotechnology Co., Shanghai, China), followed with protein quantification by using a bicinchoninic acid (BCA) kit (Beyotime Biotech-

Table I. Primers for RT-PCR.

Gene names	Forward primer (5′-3′)	Reverse primer (5′-3′)
miR-106a-5p	GCGAAAAGTGCTTACAGTGCAGG	GTGCAGGGTCCGAGGT
miR-130b-3p	ACUCUUUCCCUGUUGCACUAC	CAGUGCAAUGAUGAAGGGCAU
miR-124-3p	TGTGATGAAAGACGGCACAC	CTTCCTTTGGGTATTGTTTGG
miR-21-5p	TAGCTTATCAGACTGATGTTGA	AACGCTTCACGAATTTGCGT
CASP9	AGGTTCTCAGACCGGAAACA	CTGCATTTCCCCTCAAACTC
CASP2	ACCGTTGAGCTGTGACTATG	GTTCCGTAGCATCTGTGGATAG
SMAD5	TCAAGGATCCACCATGGCGACG	TGCACTCGAGTTATGAAACAGA
SMAD2	AAGAGAATTCACCATGGCGTCG	CTTTACTCGAGTGACATGCTTGA
STAT3	GTCTGTAGAGCCATACACCAAG	GGTAGAGGTAGACAAGTGGAGA
PTEN	ACCAGGACCAGAGGAAACCT	GCTAGCCTCTGGATTTGACG
BCL-2	ATTGTGGCCTTCTTTGAGTTCG	CATCCCAGCCTCCGTTATCC
RUNX2	CGGCCCTCCCTGAACTCT	TGCCTGCCTGGGGTCTGTA
GAPDH	GGAGTCCACTGGTGTCTTCA	GGGAACTGAGCAATTGGTGG
U6	CTCGCTTCGGCAGCACATA	ACGCTTCACGAATTTGCGT

nology Co., Shanghai, China) on the basis of its protocol. 30 μg total protein samples were added to 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) for separation, which were transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Billerica, MA, USA). After being blocked in the 5% non-fatty milk at room temperature for 1 h, these membranes were incubated with primary antibodies overnight at 4°C. All primary antibodies were bought from Abcam (Abcam, Cambridge, MA, USA), including: CASP9 (ab32539, 1:5000, 46 kDa), CASP3 (ab13847, 1:1000, 17 kDa), CASP6 (ab185645, 1:1000, 33 kDa), Bax (ab32503, 1:1000, 21 kDa), Cyclin D1 (ab134175, 1:5000, 34 kDa), Cyclin E (ab33911, 1:2000, 50 kDa), CD81 (ab79559, 1:2000, 26 kDa), CD63 (ab217345, 1:2000, 50 kDa), CD9 (ab92726, 1:2000, 25 kDa), Calreticulin (ab92516, 1:2000, 48 kDa), GAPDH (ab181602, 1:5000, 36 kDa). Then, the membranes were incubated with matched secondary antibodies (1:5000) for 1 h. Finally, protein bands were detected by using Pierce enhanced chemiluminescence (ECL) Western blot substrate (Thermo Fisher Scientific, Waltham, MA, USA) with ECL detection system (Thermo Fisher Scientific, Waltham, MA, USA).

Dual-Luciferase Reporter Gene Assay

Dual-Luciferase reporter gene assay was performed to verify whether CASP9 was a target gene of miR-106a-3p. The potential 3'UTR binding sequence was predicted by TargetScan and miRDB databases, the wild type CASP9 sequence (WT-CASP9) and mutant CASP9 sequence (MUT-CASP9) were synthesized and constructed into pmiR-GLO (Promega, Madison, WI, USA). VSMCs were seeded on 48-well plates for 24 h, miR-106a-3p mimic or miR-mimic NC was co-transfected into VSMCs with pGL3-WT-CASP9 or MUT-CASP9, the control Luciferase reporter plasmid was transfected with Lipofectamine 2000 into VSMCs for other 24 h. Finally, the cells were lysed, and the activities of Firefly Luciferase and Renilla Luciferase were measured by using a Dual-Luciferase reporter assay (Promega, Madison, WI, USA). Data were normalized to the Renilla Luciferase activity and the relative activities of luciferase were calculated.

Statistical Analysis

The data was analyzed by SPSS 19.0 (SPSS Inc., Armonk, NY, USA) and GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA). Significance between groups was analyzed by one-way analysis of variance (ANOVA) or two-way ANOVA and SNK method was used after ANOVA analysis. p-value<0.05 was considered statistically significant.

Results

Ox-LDL Induced THP-1 Promoted Cell Proliferation and Repressed Cell Apoptosis of Human VSMCs

To determine whether ox-LDL induced THP-1 could regulate the functions of human VSMCs, normal THP-1 and ox-LDL induced THP-1 were added into VSMCs for 48 h. After that, CCK-8 assay and flow cytometric analysis were performed to evaluate cell proliferation and apoptosis. Results showed that cell viability was significantly promoted after co-culturing with ox-LDL induced THP-1 cells (Figure 1A) (p<0.01). Moreover, the cell apoptotic rate was reduced (Figure 1B, C) (p<0.01). Some proliferation and apoptosis associated protein expressions were detected, and results showed that protein levels of Cyclin D1 and Cyclin E were increased, while Bax, CASP3 and CASP6 were repressed (Figure 1D, E) (p<0.01). These data revealed that ox-LDL induced THP-1 could promote cell proliferation and repress cell apoptosis in VSMCs.

Exosomes Were Extracted from THP-1 and ox-LDL Induced THP-1

Exosomes have been demonstrated as vesicles that can be important regulators and play some critical roles in biological behaviors in AS[15-18]. Previous results showed that ox-LDL induced THP-1 promoted cell proliferation and inhibited cell apoptosis in VSMCs and, herein, we investigated whether exosomes released by ox-LDL induced THP-1 might contribute to the effects. Exosomes were extracted from normal THP-1 and ox-LDL induced THP-1, and the transmission electron microscopic morphological analysis was performed to determine the exosomes, which were about 100 nm in diameter (Figure 2A). Besides, WB was performed to detect protein expressions in exosomes. CD81, CD63 and CD9 were known as markers for exosomes, and Calreticulin was a marker for lysates. Results showed that CD81, CD63 and CD9 were highly expressed while Calreticulin was little expressed in exosomes (Figure 2B), which indicated that the isolated particles were exosomes from THP-1 and ox-LDL induced THP-1.

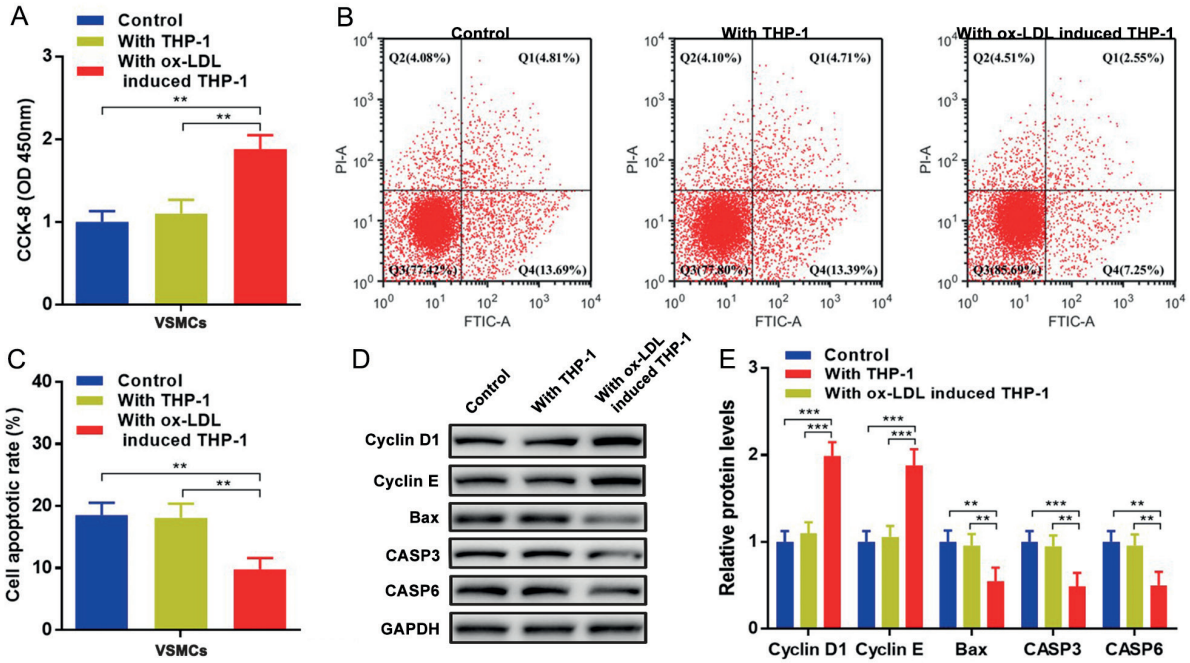

Figure 1. Ox-LDL induced THP-1 promoted cell proliferation and repressed cell apoptosis of human VSMCs. THP-1 or ox-LDL induced THP-1 was added into human VSMCs for 48 h. **A,** CCK-8 assay was performed to detect cell viability. **B-C,** Flow cytometric analysis was used to evaluate cell apoptosis. **D-E,** Western blot (WB) was performed to detect the protein expressions of Cyclin D1, Cyclin E, Bax, CASP3 and CASP6. **$p<0.01$, ***$p<0.001$.

Exosomes From ox-LDL Induced THP-1 Promoted Cell Proliferation and Repressed Cell Apoptosis of VSMCs

To further confirm whether the exosomes from ox-LDL induced THP-1 could affect cell proliferation and apoptosis in VSMCs and play some roles in AS, exosomes were separately isolated from THP-1 and ox-LDL induced THP-1 in exosome-free media, which were then added into VSMCs for 48 h. Results showed that exosomes from ox-LDL induced THP-1 could promote cell viability and repress cell apoptotic rate (Figure 3A-C) ($p<0.01$). Furthermore, WB demonstrated that the protein levels of Cyclin D1 and Cyclin E were significantly increased, while Bax, CASP3 and CASP6 were repressed (Figure 3D, E) ($p<0.01$). These data indicated that exosomes from ox-LDL induced THP-1 could affect cell proliferation and apoptosis in VSMCs.

Ox-LDL Induced THP-1 Delivered MiR-106a-3p to VSMCs Through Exosomes

Exosomes are small particles that contain miRNAs and mRNAs, which may regulate cellular functions in diseases[19-21], and we assessed whether miRNAs could be delivered to VSMCs through exosomes. To investigate the mechanism that exosomes from ox-LDL induced THP-1 could affect cell proliferation and apoptosis in VSMCs, we detected some expressions of miRNAs in exosomes that were associated with cell proliferation, including miR-106a-3p[22,25], miR-130b-3p[26], miR-205-5p[27] and miR-652-3p[28,29]. The results showed that miR-106a-3p was significantly increased in exosomes from ox-LDL induced THP-1 (Figure 4A) ($p<0.001$), while no significant differences have been found in other three miRNAs (Figure 4B-D) ($p>0.05$). In addition, exosomes were added into VSMCs for 48 h, then, their expressions were detected by RT-PCR. Results showed that miR-106a-3p was significantly increased after co-culturing with exosomes from ox-LDL induced THP-1 (Figure 4E) ($p<0.01$), but not in other three miRNAs (Figure 4F-H) ($p>0.05$). These data suggested that ox-LDL induced THP-1 delivered miR-106a-3p to VSMCs and played some roles through exosomes.

MiR-106a-3p Overexpression Promoted Cell Proliferation and Repressed Cell Apoptosis in VSMCs

To further explore the functions of miR-106a-3p in VSMCs and contributions to AS, the miR-106a-3p mimic or miR-mimic NC was respec-

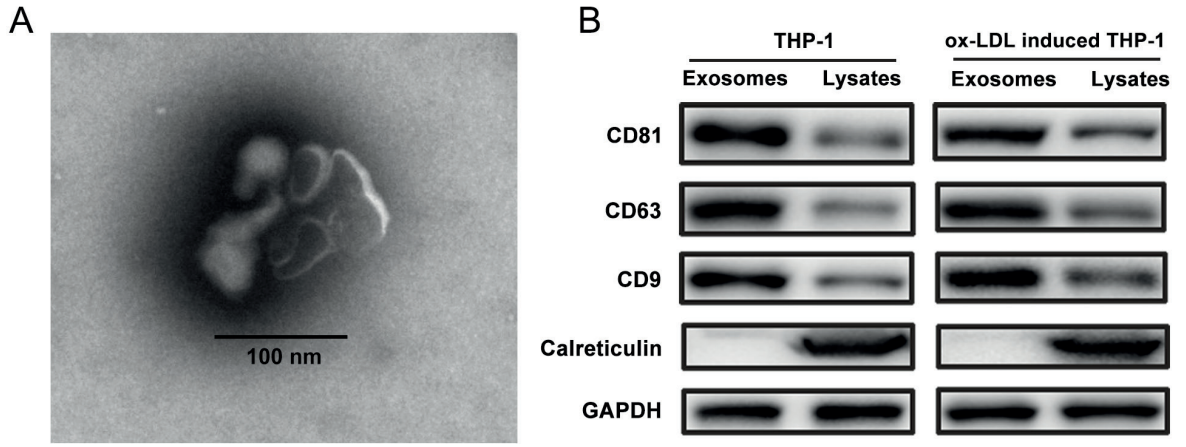

Figure 2. Exosomes were extracted from THP-1 and ox-LDL induced THP-1. **A,** Transmission electron microscopic morphological analysis of exosomes isolated from THP-1 and ox-LDL induced THP-1. Scale bar=100 nm. **B,** Exosomes and lysates were extracted, markers (CD81, CD63 and CD9) for exosomes and Calreticulin for lysates were detected by WB.

tively transfected into VSMCs, and miR-106a-3p expression was significantly increased after that (Figure 5A) (p<0.001), which indicated that miR-106a-3p was successfully transfected into VSMCs. Furthermore, CCK-8 assay showed that miR-106a-3p overexpression significantly promoted VSMCs proliferation after 72 h (Figure 5B) (p<0.001). Moreover, flow cytometric analysis showed that miR-106a-3p overexpression reduced cell apoptotic rate of VSMCs (Figure 5C) (p<0.001). In addition, the protein levels of Cyclin D1 and Cyclin E were significantly increased,

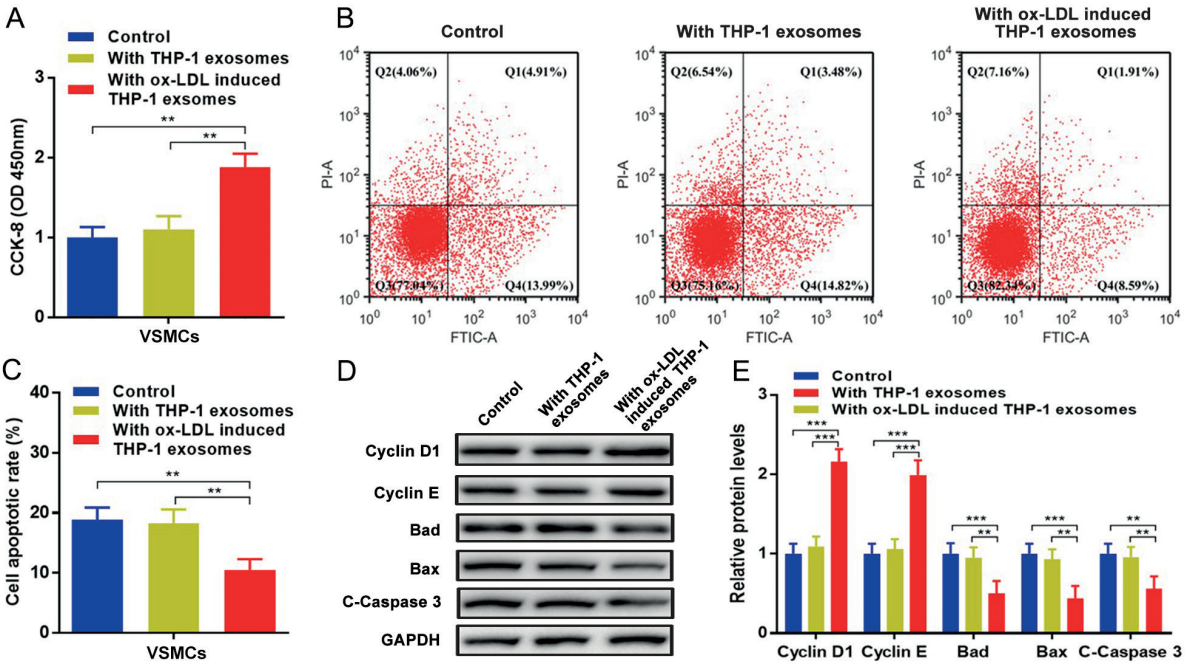

Figure 3. Exosomes from ox-LDL induced THP-1 promoted cell proliferation and repressed cell apoptosis of VSMCs. Exosomes were separately isolated from THP-1 and ox-LDL induced THP-1, which were then added into VSMCs for 48 h. **A,** CCK-8 assay was performed to detect cell viability. **B-C,** Flow cytometric analysis was used to evaluate cell apoptosis. **D-E,** WB was performed to detect the protein expressions of Cyclin D1, Cyclin E, Bax, CASP3 and CASP6. **p<0.01, ***p<0.001.

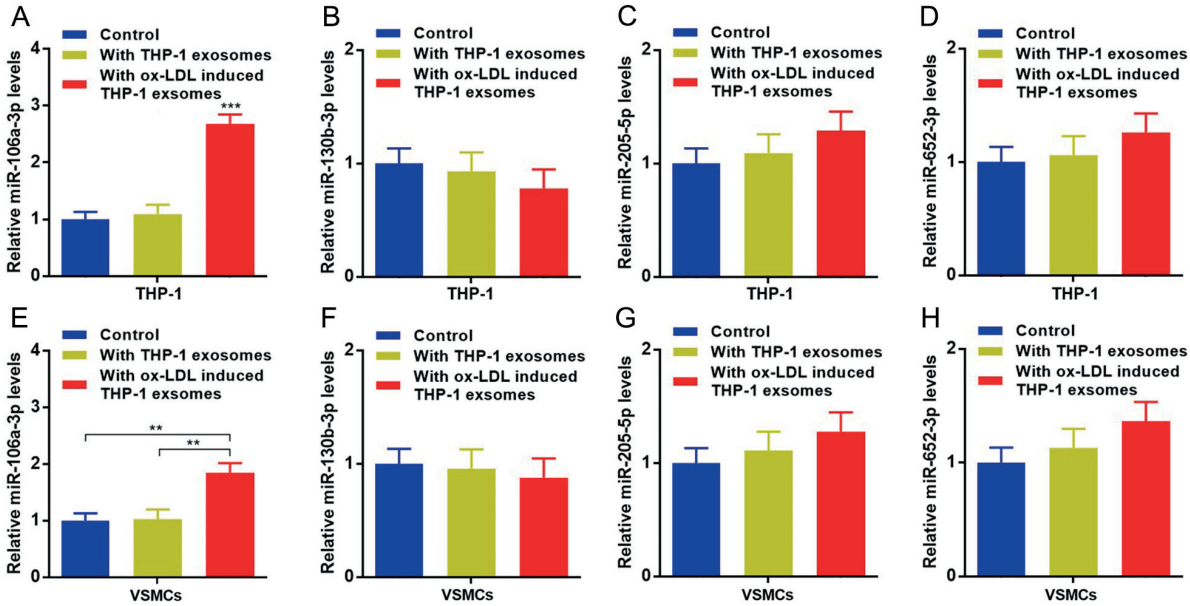

Figure 4. Ox-LDL induced THP-1 delivered miR-106a-3p to VSMCs through exosomes. **A-D,** Some miRNA levels were detected in exosomes by RT-PCR. **E-H,** Some miRNAs expressions were detected in VSMCs after co-culturing with exosomes from THP-1 or ox-LDL induced THP-1 by RT-PCR. $^{**}p<0.01$, $^{***}p<0.001$.

while Bax, CASP3 and CASP6 were repressed (Figure 5D, E) ($p<0.01$). These results indicated that miR-106a-3p overexpression promoted cell proliferation and repressed cell apoptosis in VSMCs.

MiR-106a-3p Could Directly Target at CASP9 in VSMCs

To explore the mechanism of miR-106a-3p in regulating cell proliferation and apoptosis in VSMCs, bioinformatics analysis was performed and target genes of miR-106a-3p were predicted by TargetScan and miRDB, and common genes were identified (Figure 6A). CASP9, CASP2, SMAD2, SMAD5, STAT3, PTEN, BCL-2 and RUNX2 were identified as potential target genes, which were associated with cell proliferation and apoptosis. MiR-106a-3p mimic or miR-mimic NC was respectively transfected into VSMCs, and then, these genes were detected by RT-PCR, which showed that the mRNA and protein levels of CASP9 were remarkably repressed, but not in other genes (Figure 6B, C) ($p<0.01$). To confirm whether miR-106a-3p could directly bind with CASP9, the WT-CASP9 and MUT-CASP9 vectors were constructed (Figure 6D) and the Luciferase gene reporter assay was performed. Results showed that the relative Luciferase activity in VSMCs co-transfected with WT-CASP9 and miR-106a-3p mimic was repressed compared with that in VSMCs with miR-mimic NC, while it was reversed in VSMCs co-transfected with MUT-CASP9 and miR-106a-3p mimic (Figure 6E) ($p<0.01$). These results demonstrated that miR-106a-3p could directly targeted at binding with CASP9 in VSMCs.

Blocking Exosomes From ox-LDL Induced THP-1 Reduced Cell Proliferation in VSMCs

Above results showed that ox-LDL induced THP-1 could promote cell proliferation and inhibit cell apoptosis *via* exosomes. Further, we investigated whether blocking exosome secretion by ox-LDL induced THP-1 could prevent the effects in VSMCs. GW4869 has been demonstrated to block exosome secretion in cells; as a result, we used GW4869 to block exosome secretion by ox-LDL induced THP-1 and detected the effects on cell proliferation and apoptosis in VSMCs. Results showed that GW4869 decreased exosome released by ox-LDL induced THP-1 and miR-106a-3p was reduced (Figure 7A) ($p<0.01$). Moreover, cell viability was repressed, and cell apoptotic rate was increased after adding blocking exosomes (Figure 7B-D) ($p<0.01$). Protein levels of Bax, CASP9, CASP3 and CASP6 were increased, while Cyclin D1 and Cyclin E were significantly

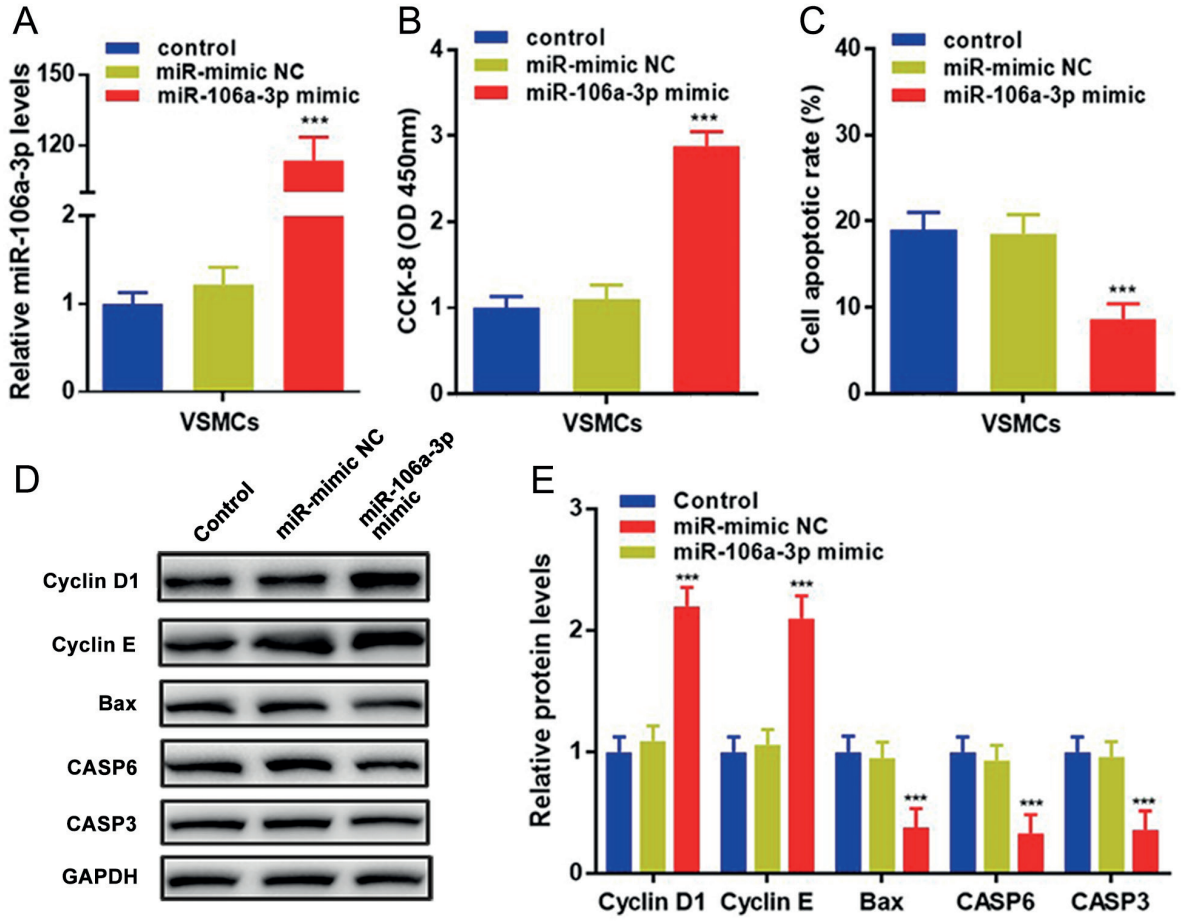

Figure 5. MiR-106a-3p overexpression promoted cell proliferation and repressed cell apoptosis in VSMCs. MiR-106a-3p mimic or miR-mimic NC was respectively transfected into VSMCs for 72 h. **A,** CCK-8 assay was performed to detect cell viability. **B-C,** Flow cytometric analysis was used to evaluate cell apoptosis. **D-E,** WB was performed to detect the protein expressions of Cyclin D1, Cyclin E, Bax, CASP3 and CASP6. ***p<0.001.

repressed (Figure 7E, F) (p<0.01). Collectively, ox-LDL induced THP-1 exosomes were the critical regulator for VSMCs and blocking exosomes secretion from ox-LDL induced THP-1 reduced cell proliferation in VSMCs.

Discussion

AS is an inflammatory disease which is a leading cause of mortality worldwide[1-4]. So far, the treatment and prognosis of AS patients were not well satisfied, so more understanding and new strategies for treating AS are needed. Exosomes are small membranous EVs that transfer miRNAs, mRNAs and proteins to target cells and regulate the biological functions and development of AS[15-18]. Therefore, in this study, we wanted to investigate potential functions of exosomes derived from ox-LDL exposed macrophages. Our previous results found that ox-LDL induced macrophages could transfer some molecules to promote cell proliferation and repress cell apoptosis of human VSMCs. However, the detailed mechanism remained unknown, therefore, we wondered if the effects were regulated by exosomes from ox-LDL exposed macrophages.

Then, we isolated the exosomes from THP-1 and ox-LDL induced THP-1 by using ultracentrifugation, which were confirmed by WB assay and transmission electron microscopy. Furthermore, the isolated exosomes from THP-1 and ox-LDL induced THP-1 were added into VSMCs for 48 h, we found that exosomes from ox-LDL induced THP-1 could promote cell proliferation and repress cell apoptosis of VSMCs, which revealed

that ox-LDL exposed macrophages might affect the functions of VAMCs by releasing exosomes. However, the detailed mechanisms remained unclear.

Exosomes are small particles that contain miRNAs and mRNAs, which can be transferred into other kinds of cells and regulate biological functions in diseases[19-21]. In our study, we found that miR-106a-3p was increased in exosomes from ox-LDL induced THP-1 and the exosomes could be transferred into VSMCs and the miR-106a-3p expression was increased in VSMCs. Further study indicated that miR-106a-3p overexpression promoted cell proliferation and repressed cell apoptosis in VSMCs. Ox-LDL could lead to induce the proliferation of VSMCs and endothelial cells (ECs), thus promoting the formation of vascular atherosclerotic lesions[30-32]. VSMCs are a kind of critical components of blood vessels, and their proliferation leads to the intimal thickening and promote the development of AS[32-34]. In this study, we found that the increased miR-106a-3p in VSMCs transferred by ox-LDL induced THP-1 through exosomes could promote cell proliferation and repress cell apoptosis of VSMCs; as a result, it could play important roles in the atherosclerotic process. However, these mechanisms remained unknown.

Caspase-9 belongs to the caspase family, which is an enzyme that is encoded by the CASP9 gene and it is an initiator caspase to promote cell apoptosis[35-38]. The activation of caspase-9 will go on to activate cleaved caspase-3 and caspase-6, thereby initiating the caspase cascade and cell apoptosis[35-38]. To explore the mechanism of miR-106a-3p in regulating cell proliferation and apoptosis of VSMCs, we used TargetS-

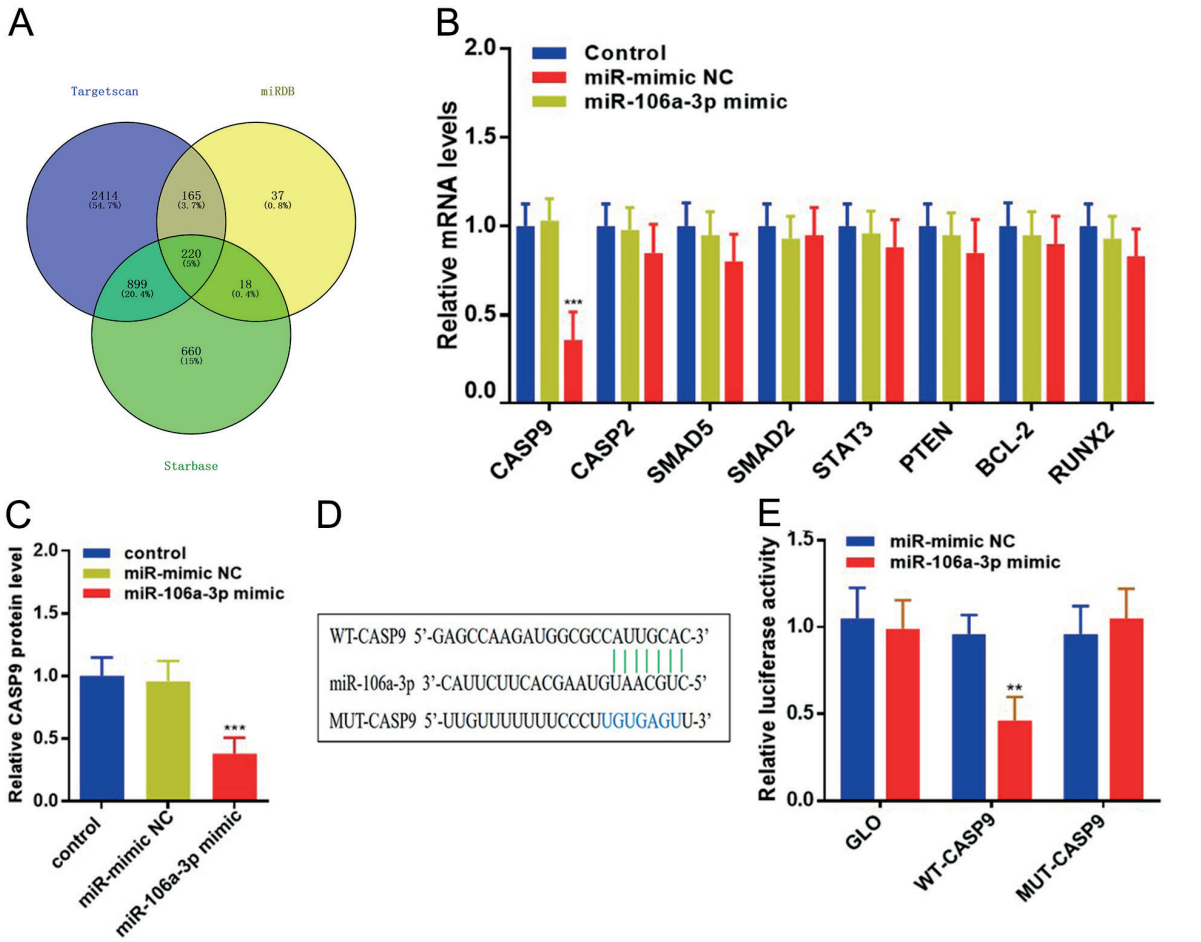

Figure 6. MiR-106a-3p could directly target at CASP9 in VSMCs. **A,** miRDB and TargetScan were performed to predict target genes. **B,** The mRNA expressions of some potential genes were detected by RT-PCR. **C,** Protein expression of CASP9 was detected by WB. **D,** Potential binding sites between miR-106a-3p and CASP9 were predicted by TargetScan database and vectors were constructed. **E,** Luciferase reporter assay was performed to prove the binding site. $^{**}p<0.01$, $^{***}p<0.001$.

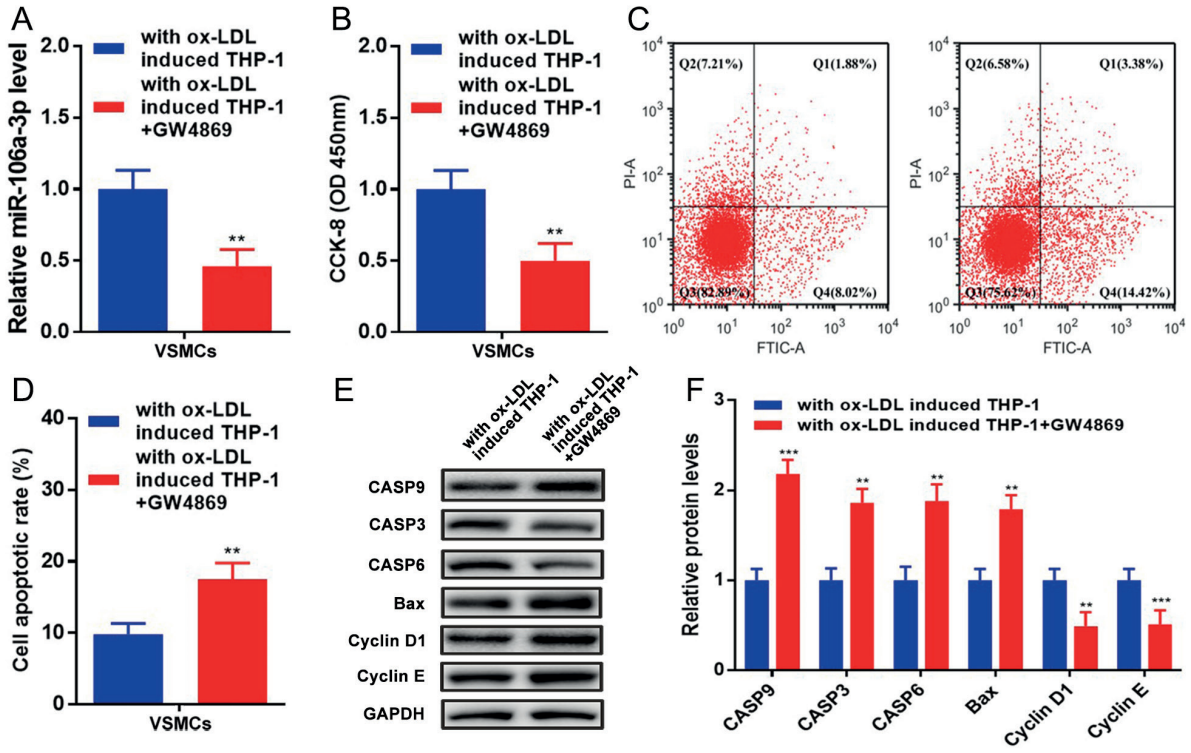

Figure 7. Blocking exosomes from ox-LDL induced THP-1 and reduced cell proliferation in VSMCs. GW4869 was used to block exosome secretion by ox-LDL induced THP-1. **A,** MiR-106a-3p expression was detected by RT-PCR. **B,** CCK-8 assay was performed to detect cell viability. **C-D,** Flow cytometric analysis was used to evaluate cell apoptosis. **E-F,** WB was performed to detect the protein expressions of CASP9, CASP3, CASP6, Bax, Cyclin D1 and Cyclin E. **p<0.01, ***p<0.001.

can and miRDB to predict target genes for miR-106a-3p, and Luciferase gene reporter assay showed that miR-106a-3p could directly bind with CASP9, which were important in the caspase-dependent apoptotic cell death of AS[39,40].

Finally, we used GW4869, an exosome inhibitor, to block exosome secretion from ox-LDL induced THP-1 and observed the effects of exosomes-mediated miR-106a-3p on cell proliferation and apoptosis in VSMCs. We found that blocking exosomes from ox-LDL induced THP-1 could reduce the cell proliferation and promote cell apoptosis in VSMCs, which indicated that ox-LDL induced THP-1 exosomes were pivotal for the regulation of cell proliferation and apoptosis in VSMCs.

Therefore, our study firstly showed that miR-106a-3p was upregulated in exosomes from ox-LDL induced THP-1, which could regulate cell proliferation and apoptosis in VSMCs. We found a novel pathway that exosomes-mediated miR-106a-3p could directly bind with CASP9 and might regulate the development of AS.

Conclusions

Taken together, for the first time, this study discovered that miR-106a-3p was upregulated in exosomes from THP-1 induced by ox-LDL, which could be delivered to VSMCs and regulate cell proliferation and cell apoptosis of VSMCs. Surprisingly, we demonstrated that the exosomes-mediated miR-106a-3p could directly bind with the downstream target CASP9 and repress Caspase signaling pathway in VSMCs, which might provide a potential target and new insight for treating AS.

Conflict of Interest

The Authors declare that they have no conflict of interests.

References

1) Hedin U, Matic LP. Recent advances in therapeutic targeting of inflammation in atherosclerosis. J Vasc Surg 2019; 69: 944-951.

2) Herrington W, Lacey B, Sherliker P, Armitage J, Lewington S. Epidemiology of atherosclerosis and the potential to reduce the global burden of atherothrombotic disease. Circ Res 2016; 118: 535-546.
3) Lu Y, Ballew SH, Tanaka H, Szklo M, Heiss G, Coresh J, Matsushita K. 2017 ACC/AHA blood pressure classification and incident peripheral artery disease: The Atherosclerosis Risk in Communities (ARIC) study. Eur J Prev Cardiol 2020; 27: 51-59.
4) Mozaffarian D, Benjamin EJ, Go AS, Arnett DK, Blaha MJ, Cushman M, de Ferranti S, Després JP, Fullerton HJ, Howard VJ, Huffman MD, Judd SE, Kissela BM, Lackland DT, Lichtman JH, Lisabeth LD, Liu S, Mackey RH, Matchar DB, McGuire DK, Mohler ER 3rd, Moy CS, Muntner P, Mussolino ME, Nasir K, Neumar RW, Nichol G, Palaniappan L, Pandey DK, Reeves MJ, Rodriguez CJ, Sorlie PD, Stein J, Towfighi A, Turan TN, Virani SS, Willey JZ, Woo D, Yeh RW, Turner MB; American Heart Association Statistics Committee and Stroke Statistics Subcommittee. Heart disease and stroke statistics--2015 update: a report from the American Heart Association. Circulation 2015; 131: e29-322.
5) Li Y, Niu X, Xu H, Li Q, Meng L, He M, Zhang J, Zhang Z, Zhang Z. VX-765 attenuates atherosclerosis in ApoE deficient mice by modulating VSMCs pyroptosis. Exp Cell Res 2020; 389: 111847.
6) Xu H, Jiang J, Chen W, Li W, Chen Z. Vascular macrophages in atherosclerosis. J Immunol Res 2019; 2019: 4354786.
7) Moroni F, Ammirati E, Norata GD, Magnoni M, Camici PG. The role of monocytes and macrophages in human atherosclerosis, plaque neoangiogenesis, and atherothrombosis. Mediators Inflamm 2019; 2019: 7434376.
8) Gao S, Zhao D, Qi Y, Wang W, Wang M, Sun J, Liu J, Li Y, Liu J. Circulating oxidized low-density lipoprotein levels independently predict 10-year progression of subclinical carotid atherosclerosis: a community-based cohort study. J Atheroscler Thromb 2018; 25: 1032-1043.
9) Tsilingiri K, de la Fuente H, Relano M, Sanchez-Diaz R, Rodriguez C, Crespo J, Sanchez-Cabo F, Dopazo A, Alonso-Lebrero JL, Vara A, Vazquez J, Casasnovas JM, Alfonso F, Ibanez B, Fuster V, Martinez-Gonzalez J, Martin P, Sanchez-Madrid F. Oxidized low-density lipoprotein receptor in lymphocytes prevents atherosclerosis and predicts subclinical disease. Circulation 2019; 139: 243-255.
10) Itabe H. Oxidized low-density lipoprotein as a biomarker of in vivo oxidative stress: from atherosclerosis to periodontitis. J Clin Biochem Nutr 2012; 51: 1-8.
11) Yang H, Mohamed AS, Zhou SH. Oxidized low density lipoprotein, stem cells, and atherosclerosis. Lipids Health Dis 2012; 11: 85.
12) van Niel G, D'Angelo G, Raposo G. Shedding light on the cell biology of extracellular vesicles. Nat Rev Mol Cell Biol 2018; 19: 213-228.
13) Dhondt B, Van Deun J, Vermaerke S, de Marco A, Lumen N, De Wever O, Hendrix A. Urinary extracellular vesicle biomarkers in urological cancers: From discovery towards clinical implementation. Int J Biochem Cell Biol 2018; 99: 236-256.
14) Lopes-Rodrigues V, Di Luca A, Sousa D, Seca H, Meleady P, Henry M, Lima RT, O'Connor R, Vasconcelos MH. Multidrug resistant tumour cells shed more microvesicle-like EVs and less exosomes than their drug-sensitive counterpart cells. Biochim Biophys Acta 2016; 1860: 618-627.
15) Li J, Xue H, Li T, Chu X, Xin D, Xiong Y, Qiu W, Gao X, Qian M, Xu J, Wang Z, Li G. Exosomes derived from mesenchymal stem cells attenuate the progression of atherosclerosis in ApoE(-/-) mice via miR-let7 mediated infiltration and polarization of M2 macrophage. Biochem Biophys Res Commun 2019; 510: 565-572.
16) Lu X. The role of exosomes and exosome-derived microRNAs in atherosclerosis. Curr Pharm Des 2017; 23: 6182-6193.
17) Wang Y, Xie Y, Zhang A, Wang M, Fang Z, Zhang J. Exosomes: an emerging factor in atherosclerosis. Biomed Pharmacother 2019; 115: 108951.
18) Zhang YG, Song Y, Guo XL, Miao RY, Fu YQ, Miao CF, Zhang C. Exosomes derived from oxLDL-stimulated macrophages induce neutrophil extracellular traps to drive atherosclerosis. Cell Cycle 2019; 18: 2674-2684.
19) Shukla GC, Singh J, Barik S. MicroRNAs: processing, maturation, target recognition and regulatory functions. Mol Cell Pharmacol 2011; 3: 83-92.
20) Joppich M, Weber C, Zimmer R. Using context-sensitive text mining to identify miRNAs in different stages of atherosclerosis. Thromb Haemost 2019; 119: 1247-1264.
21) Mao Z, Wu F, Shan Y. Identification of key genes and miRNAs associated with carotid atherosclerosis based on mRNA-seq data. Medicine (Baltimore) 2018; 97: e9832.
22) Yang XZ, Cheng TT, He QJ, Lei ZY, Chi J, Tang Z, Liao QX, Zhang H, Zeng LS, Cui SZ. LINC01133 as ceRNA inhibits gastric cancer progression by sponging miR-106a-3p to regulate APC expression and the Wnt/beta-catenin pathway. Mol Cancer 2018; 17: 126.
23) Guo W, Li W, Yuan L, Mei X, Hu W. MicroRNA-106a-3p induces apatinib resistance and Activates Janus-Activated Kinase 2 (JAK2)/Signal Transducer and Activator of Transcription 3 (STAT3) by targeting the SOCS system in gastric cancer. Med Sci Monit 2019; 25: 10122-10128.
24) Uhr K, Prager-van der Smissen WJC, Heine AAJ, Ozturk B, van Jaarsveld MTM, Boersma AWM, Jager A, Wiemer EAC, Smid M, Foekens JA, Martens JWM. MicroRNAs as possible indicators of drug sensitivity in breast cancer cell lines. PLoS One 2019; 14: e0216400.
25) Lu J, Mu X, Yin Q, Hu K. MiR-106a contributes to prostate carcinoma progression through PTEN. Oncol Lett 2019; 17: 1327-1332.
26) Xu Z, Han Y, Liu J, Jiang F, Hu H, Wang Y, Liu Q, Gong Y, Li X. MiR-135b-5p and MiR-499a-3p promote cell proliferation and migration in atherosclerosis by directly targeting MEF2C. Sci Rep 2015; 5: 12276.
27) Meng X, Yin J, Yu X, Guo Y. MicroRNA-205-5p promotes unstable atherosclerotic plaque formation in vivo. Cardiovasc Drugs Ther 2020; 34: 25-39.

28) Huang R, Hu Z, Cao Y, Li H, Zhang H, Su W, Xu Y, Liang L, Melgiri ND, Jiang L. MiR-652-3p inhibition enhances endothelial repair and reduces atherosclerosis by promoting Cyclin D2 expression. EBioMedicine 2019; 40: 685-694.
29) Vegter EL, Ovchinnikova ES, van Veldhuisen DJ, Jaarsma T, Berezikov E, van der Meer P, Voors AA. Low circulating microRNA levels in heart failure patients are associated with atherosclerotic disease and cardiovascular-related rehospitalizations. Clin Res Cardiol 2017; 106: 598-609.
30) Wang C, Zhang Y, Yang Q, Yang Y, Gu Y, Wang M, Wu K. A novel cultured tissue model of rat aorta: VSMC proliferation mechanism in relationship to atherosclerosis. Exp Mol Pathol 2007; 83: 453-458.
31) Stintzing S, Ocker M, Hartner A, Amann K, Barbera L, Neureiter D. Differentiation patterning of vascular smooth muscle cells (VSMC) in atherosclerosis. Virchows Arch 2009; 455: 171-185.
32) Zhang MJ, Zhou Y, Chen L, Wang X, Long CY, Pi Y, Gao CY, Li JC, Zhang LL. SIRT1 improves VSMC functions in atherosclerosis. Prog Biophys Mol Biol 2016; 121: 11-15.
33) Chen L, Zheng SY, Yang CQ, Ma BM, Jiang D. MiR-155-5p inhibits the proliferation and migration of VSMCs and HUVECs in atherosclerosis by targeting AKT1. Eur Rev Med Pharmacol Sci 2019; 23: 2223-2233.
34) Xu L, Hao H, Hao Y, Wei G, Li G, Ma P, Xu L, Ding N, Ma S, Chen AF, Jiang Y. Aberrant MFN2 transcription facilitates homocysteine-induced VSMCs proliferation via the increased binding of c-Myc to DNMT1 in atherosclerosis. J Cell Mol Med 2019; 23: 4611-4626.
35) Brentnall M, Rodriguez-Menocal L, De Guevara RL, Cepero E, Boise LH. Caspase-9, caspase-3 and caspase-7 have distinct roles during intrinsic apoptosis. BMC Cell Biol 2013; 14: 32.
36) Chang-Qing F, Yi L, De-Guang W, Qing-Bin S, Xiang-Min H, Na T, Jian-Hua L. Immune clearance gastric carcinoma cells in ascites by activating caspase-9-induced apoptosis. APMIS 2011; 119: 173-179.
37) Kuida K. Caspase-9. Int J Biochem Cell Biol 2000; 32: 121-124.
38) Liu LS, Bai XQ, Gao Y, Wu Q, Ren Z, Li Q, Pan LH, He NY, Peng J, Tang ZH. PCSK9 promotes oxLDL-induced PC12 cell apoptosis through the Bcl-2/Bax-Caspase 9/3 signaling pathway. J Alzheimers Dis 2017; 57: 723-734.
39) Chao ML, Guo J, Cheng WL, Zhu XY, She ZG, Huang Z, Ji Y, Li H. Loss of caspase-activated dnase protects against atherosclerosis in apolipoprotein e-deficient mice. J Am Heart Assoc 2016; 5. pii: e004362.
40) Tajbakhsh A, Kovanen PT, Rezaee M, Banach M, Moallem SA, Sahebkar A. Regulation of efferocytosis by caspase-dependent apoptotic cell death in atherosclerosis. Int J Biochem Cell Biol 2020; 120: 105684.