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Original Article

Naosuikang-attenuated inflammatory response in atherosclerotic mice is associated with exosomal miRNA

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Abstract: Atherosclerosis is the pathophysiological basis of circulatory system diseases. Inflammation is not only a key indicator, but is also the driving force behind these diseases. Atherosclerosis is therefore treated using anti-inflammatory strategies. Chinese herbal medicine is widely used in Asia, China in particular. Naosuikang is a Chinese herbal medicine that has been used clinically, and its clinical efficacy has been confirmed, but its specific mechanism needs further clarification. Exosomes are the transmitters of signals between cells, and they participate in the occurrence and development of inflammation. We hypothesized that Naosuikang uses exosomal microRNA (miRNA) in its role as a treatment for atherosclerosis. In this project, we used atherosclerotic mice as an animal model, treating them with various doses of Naosuikang and observing the external effects of miRNAs on the body. This research will provide insight into the mechanism of action of Naosuikang.

Keywords: Naosuikang, atherosclerotic, exosomes, miRNA

Introduction

Atherosclerosis (AS) is a major contributor to cardiovascular diseases, a leading cause of morbidity and mortality worldwide [1, 2]. Inflammation is not only the key indicator of AS but also drives disease progression. Therefore, a promising strategy for AS is an anti-inflammatory treatment regimen [3]. Exosomes are membrane-bound nanocapsule structures secreted by cells, and they have been shown to regulate the function and phenotype of receptor cells through the transfer of related lipids, protein, RNA, and DNA [4]. In inflammatory diseases such as AS and other vascular diseases, exosomes derived from endothelial cells, vascular smooth muscle cells, macrophages, and other circulating immune cells have primarily pro-inflammatory properties [5]. The pro-inflammatory effects of exosomes have been confirmed in other pathological conditions as well [6]. Studies suggest that strategies to reduce exosome secretion can have protective effects in inflammatory diseases [7, 8]. Despite the rapid emergence of new evidence of the role of exosome communication in inflammatory diseases, little progress has been made in identifying candidate drugs that can regulate exosome production and secretion. Single-stranded non-coding small RNAs, known as microRNAs (miRNAs), participate in nearly all stages of AS; from its beginning through disease progression and until the final clinical complications of the disease [9]. In the atherosclerotic ApoE-/- mouse model, abnormal expressions of miRNAs are observed in atherosclerotic lesions and lipopolysaccharide-activated macrophages, and evidence that exosomes participate in AS has been found [10, 11].

Naosuikang was developed by Shenzhen Hospital of Traditional Chinese Medicine, a facility affiliated with Guangzhou University of Traditional Chinese Medicine (Z20070561 for Cantonese medicine). Naosuikang consists of astragalus root, kudzu vine root, glossy privet fruit, gastrodia elata, ligusticum wallichii, salvia miltiorrhiza, and other medicinal Chinese

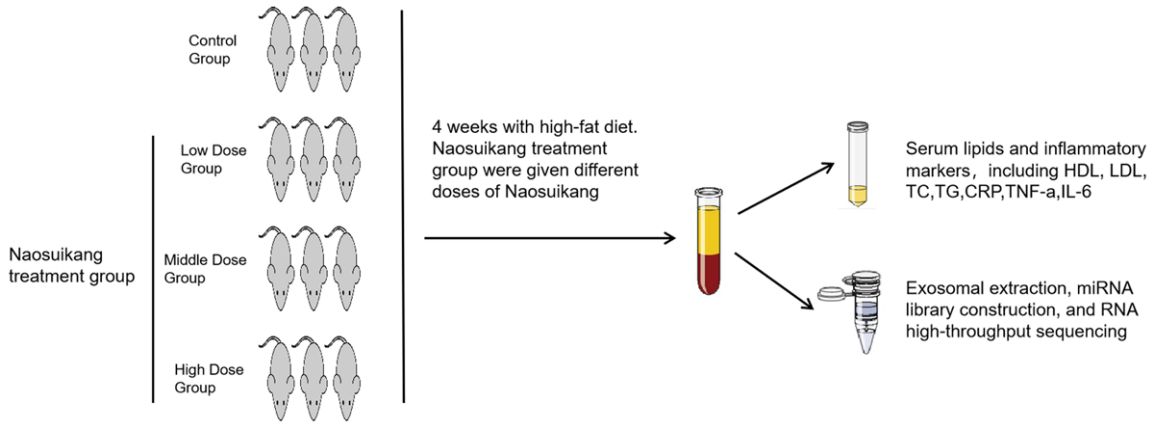


Figure 1. Flow chart of exosomal RNA sequencing in atherosclerotic mice. HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; CRP, c-reactive protein; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6; miRNA, microRNA.

herbs. Clinical studies have found that Naosuikang improves the situational memory of rats by adjusting their oxidative stress levels [12]. Based on its composition and data from recent studies [13, 14], we speculate that Naosuikang participates in regulating the inflammatory response in AS by affecting miRNA levels in the exosomes, thus slowing the AS disease process. Our goal was to investigate the effects of Naosuikang on mouse exosomal miRNAs in a mouse model of AS.

Materials and methods

Laboratory animals

Male ApoE-/-mice were purchased from Beijing Vital River Laboratory Animal Technology Co., Ltd. then divided into four groups (6 per group): the low-dose Naosuikang group, the medium-dose Naosuikang group, the high-dose Naosuikang group, and a control group. All mice were fed a high-fat feed (2% cholesterol, 6% lard, 10% egg yolk powder, 0.2% bile salt, and 81.8% basic feed) produced by Guangzhou Jinan University Experimental Animal Center. This study was approved by the Animal Ethics Committee of the Fourth Clinical Medical College, Guangzhou University of Chinese Medicine (approval number: ZCYA2020080232).

Treatment methods

The classic ApoE-/- mouse AS model was used, and AS was induced by feeding the high-fat feed alone for 4 weeks beginning at 6 weeks of age. The three Naosuikang groups were given 3.75 mg, 7.5 mg, or 15 mg Naosuikang at the beginning of the high-fat diet, whereas the control group was given 2 ml/kg normal saline once daily. The mice were kept at the Specific Pathogen Free Laboratory Animal Center of Jinan University, 6 to a cage. The room was kept at a constant temperature of 20°C, and artificial light and dark alternated every 12 hours. All operations conformed to the requirements of the Animal Protection and Use Committee of Guangdong Laboratory Animal Monitoring Institute. The experiment is shown in **Figure 1**.

Measuring plasma lipids

After 4 weeks of treatment, the animals were fasted for 12 hours, and a blood sample was taken from the fundus venous plexus of every animal. Samples were immediately centrifuged at 1200×g at 4°C for 10 minutes then rested for 2 hours and centrifuged at room temperature for 15 minutes. The supernatant was taken, and the plasma was analyzed for triglyceride (TG), total cholesterol (TC), low-density lipoprotein (LDL), and high-density lipoprotein (HDL) using commercial kits supplied by Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

Plasma TNF-α, IL-6, and CRP detection

Following manufacturer directions, enzyme-linked immunosorbent assay kits (Nanjing Jiancheng Bioengineering Institute) were used to test for tumor necrosis factor-alpha (TNF-α),

interleukin-6 (IL-6), and c-reactive protein (CRP) in the plasma. The absorbance of each well was measured using an enzyme labeling instrument set to a wavelength of 450 nm. The standard curve was drawn using the concentration as the abscissa and the standard absorbance value as the ordinate. The corresponding concentration was calculated using the standard curve.

ZetaView nanoparticle tracking analysis

Exosome particle sizes and concentrations were measured using nanoparticle tracking analysis (NTA) on a ZetaView PMX 110 (ParticleMetrix, Meerbusch, Germany) and the corresponding software, ZetaView 8.04.02 and camera (0.703 μm/px). Once the isolated mouse exosome samples were diluted in phosphate-buffered saline (PBS; Beyotime, Shanghai, China), particle sizes and concentrations were measured using NTA then those measures were recorded and analyzed at 10 positions, maintaining the temperature between 20°C and 30°C.

Extracting exosomal RNA and preparing the sequencing library

Using manufacturer instructions, exoEasy Maxi Kits (QIAGEN, catalog number 76064, Germantown, MD, USA) were used to isolate exosomes from the peripheral blood. An Agilent 2100 Bioanalyzer was used to analyze RNA quality, yield, and size range. Extracted RNA was used to prepare a next generation sequencing library for exosomal miRNA using a QIASeq miRNA library kit (QIAGEN, catalog number 331505, Germantown, MD, USA). Exosomal miRNA sequencing was performed using the Illumina NovaSeq 6000 system (HaploX Biotechnology Co., Ltd., Shenzhen, China) and unique molecular index counting.

Sequencing data analysis and statistical analysis

The sequencing data was processed using conventional bioinformatics analytical methods. Raw data were demultiplexed and quality trimmed using the standard Illumina bcl2fastq conversion software. The miRNAs were considered differentially expressed if |log2FC| exceeded 1 and the adjusted P<0.05. R software (version 3.5.1) statistical language and R Studio software were used for analyses. The unpaired Student *t* test was used to find significant differences between two groups, whereas a one-way analysis of variance was used to estimate differences among three or more groups. Significant differences were recognized when P<0.05.

Gene ontology and Kyoto encyclopedia of genes and genomes pathways

Candidate miRNAs were uploaded to starBase, and the common target genes were selected for further analysis. Gene ontology (GO) analysis was performed to explore the functional roles of miRNA-targeting genes in terms of biological processes, cellular components, and molecular functions. The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to explore pathways related to miRNA-targeting genes. All visualizations were carried out using R Studio software (version 1.3.1086). Significant enrichment between compared groups was recognized when P<0.05.

Results

Effects of naosuikang on plasma lipids and inflammatory factors in ApoE mice

The blood lipid determinations showed that plasma LDL, TC, and TG levels in the Naosuikang groups were less than in controls, but not to a statistically significant degree. However, the plasma HDL levels in the high-dose Naosuikang group were significantly greater than in the controls (P<0.05). The plasma inflammatory factors IL-6, TNF-α, and CRP were lower in each of the Naosuikang groups than in controls. In particular, IL-6 and CRP were significantly lower in the high-dose group (P<0.05), and TNF-α was significantly lower in the middle-dose group (P<0.05). On the other hand, while the levels of IL-6, TNF-α, and CRP were lower in the low-dose group than in controls, the difference was not statistically significant. Overall, Naosuikang produced a good anti-inflammatory effect in ApoE-/-mice fed a high-fat diet. Detailed experimental data and statistical results are shown in **Table 1**.

Characterization of isolated exosomes

Exosomes isolated from plasma were characterized and confirmed using NTA, which reve-

Table 1. Blood lipids and inflammatory indexes in mice treated with one of three dosages of Naosuikang

	Control Group	Low-Dose Group	Middle-Dose Group	High-Dose Group
HDL	1.55±0.42	1.46±0.58	1.89±0.34	2.01±0.23*
LDL	2.48±0.73	2.21±0.84	1.83±0.61	1.72±0.56
TC	6.21±0.98	5.36±0.93	5.3±0.78	5.3±0.64
TG	0.62±0.14	0.59±0.08	0.58±0.11	0.49±0.14
CRP (ng/ml)	29.6±6.1	28.2±7.0	23.2±4.5	22.5±4.3*
TNF-α (pg/ml)	68.6±9.2	64.1±8.0	58±6.7*	59.9±5.8
IL-6 (pg/ml)	420±38	411±27	385±23	376±25*

Compared with controls, *P<0.05. HDL, high-density lipoprotein; LDL, low-density lipoprotein; TC, total cholesterol; TG, triglyceride; CRP, c-reactive protein; TNF-α, tumor necrosis factor-alpha; IL-6, interleukin-6.

aled sizes ranging from ~60 nm to 210 nm in diameter. The most abundant size was 149 nm, as shown in **Figure 2**.

Differences in exosomal miRNA profiling induced by naosuikang

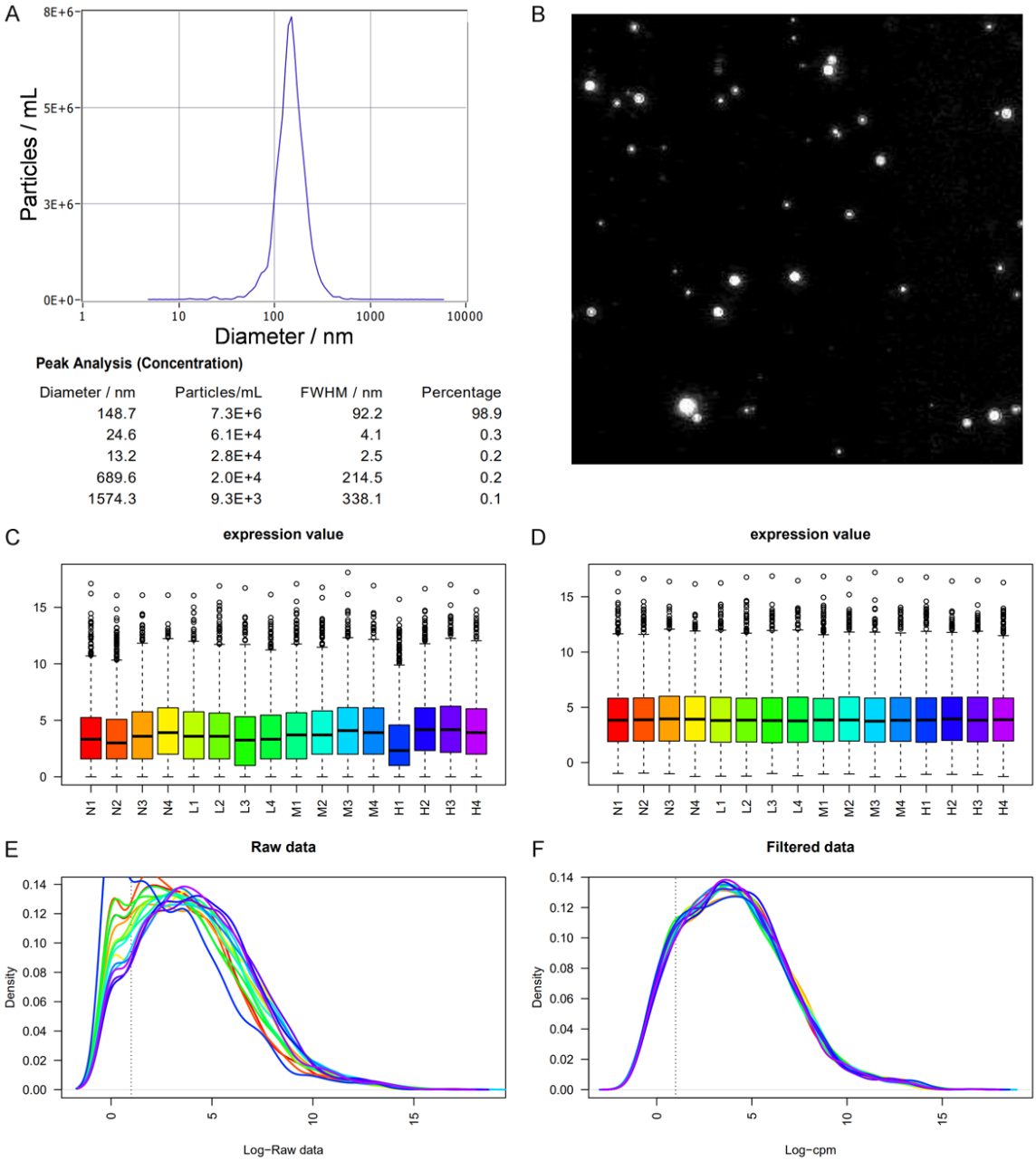
Considering the lack of significant changes in inflammatory factors and insufficient blood sample sizes in the low-dose group, no further analyses were conducted for this group. Exosomal sequencing data was processed using conventional analytical methods including demultiplexing the original data, trimming the quality, and performing downstream analyses on the standardized data. **Figure 2C-F** compares the data before and after processing. In total, 1741 mmu-miRs were identified; the most abundant among them was mmu-miR-16-5p, as shown in **Table 2**. A comparison between controls and the middle-dose group revealed 68 miRNAs that differed (**Figure 3A**); a comparison with the high-dose group revealed 87 (**Figure 3B**). The intersection between these two groups contains 23 miRNAs. Those with increased expressions in the Naosuikang groups were mmu-miR-150-3p, mmu-miR-690, mmu-miR-200b-3p, mmu-miR-335-5p, mmu-miR-206-3p, mmu-miR-378a-3p, mmu-miR-200a-3p, mmu-miR-1897-5p, mmu-miR-126a-3p, mmu-miR-1a-3p, mmu-miR-133a-3p, mmu-miR-344g-3p, mmu-miR-6980-5p, and mmu-miR-7071-5p. Conversely, those with decreased expressions were mmu-miR-322-5p, mmu-miR-205-5p, mmu-miR-27b-3p, mmu-miR-184-3p, mmu-miR-152-3p, mmu-miR-7026-3p, mmu-miR-429-3p, mmu-miR-874-3p, and mmu-miR-7067-5p. **Figure 3C** shows a heatmap of these upregulated and downregulated exosomal miRNAs.

GO terms and KEGG pathway analyses of candidate miRNA target genes

The potential biological roles of the candidate exosomal miRNAs in these mice were explored. TargetScan was used to predict potential target genes. The GO analysis included biological processes, cellular components, and molecular functions. The downstream target genes of downregulated miRNAs were enriched primarily in regulating membrane permeability, responses to radiation, endomembrane system organization, regulation of protein stability, responses to topologically incorrect proteins, and cell-substrate adhesion. The target genes of upregulated miRNAs were primarily enriched in the ephrin receptor signaling pathway, labyrinthine layer development, embryonic placenta development, regulation of translation, and tissue remodeling (**Figure 3D**, **3E**). The ten pathways most identified using KEGG pathway analysis related to target genes of downregulated miRNAs are shown in **Figure 3F**. The KEGG pathway related to target genes of upregulated miRNAs are shown in **Figure 3G**.

Discussion

Atherosclerosis is the major pathological basis for cardiovascular and cerebrovascular diseases; its formation process involves lipid infiltration, inflammation, and oxidative stress [15]. Traditional Chinese medicine theory holds that AS plaque is a visible evil in the pulse channel and can be differentiated based on “blood stasis” [16]. Because AS blood stasis is an important pathogenetic element of AS, promoting blood circulation and removing blood stasis are important therapeutic methods, and qi circulation and phlegm elimination should be included



Diameter / nm	Particles/mL	FWHM / nm	Percentage
148.7	7.3E+6	92.2	98.9
24.6	6.1E+4	4.1	0.3
13.2	2.8E+4	2.5	0.2
689.6	2.0E+4	214.5	0.2
1574.3	9.3E+3	338.1	0.1

Figure 2. Preliminary analysis of the results of exosomal identification and miRNA sequencing. A. Size distribution of the exosomes using nanoparticle tracking analysis. B. Video screenshot of exosomes. C. Exosomal miRNA high-throughput sequencing expression values before normalization. D. Exosomal miRNA high-throughput sequencing expression values after normalization. E. Exosomal miRNA high-throughput sequencing before numerical filtering. F. Exosomal miRNA high-throughput sequencing after numerical filtering.

in clinical applications [17]. Naosuikang, formulated to invigorate the spleen and kidney and eliminate phlegm and dredging collaterals, is composed of traditional Chinese medicines. Pharmacological studies have found that various components of Naosuikang play important roles in cardiovascular, cerebrovascular, and metabolic diseases, etc., but the detailed mechanism of action needs further clarification [18].

Exosomes are primarily vesicles fused by intracellular multivesicular bodies and cell membranes, and they are released into the extracel-

Table 2. Ten most abundant mouse exosomal miRNAs

Rank	Gene ID	Average Value (log2 (exp))
1	mmu-miR-16-5p	16.61339298
2	mmu-miR-21a-5p	14.02660525
3	mmu-let-7i-5p	13.73771469
4	mmu-miR-5126	13.63391585
5	mmu-let-7f-5p	13.44667397
6	mmu-miR-142a-3p	13.34598487
7	mmu-miR-6406	13.27105646
8	mmu-let-7a-5p	13.01974153
9	mmu-miR-126a-3p	13.00251903
10	mmu-let-7c-5p	12.98393979
Total	1741	

lular matrix [19]. In this study, we found that exosomes are about 140 nm in diameter, slightly larger than what is reported in the literature. This difference could be explained by the extraction methods. Current beliefs hold that almost all eukaryotic cells, including some microorganisms, can produce exosomes [20]. It has been found that exosomes derived from cellular vesicles can carry an abundance of specific information for long-distance transportation *in vivo* [21]. In addition, to achieve intercellular information transmission, the membrane structures of exosomes must carry a variety of antigens and antibody molecules, thus participating in a variety of physiological and pathological processes [22, 23].

Naosuikang has been shown to improve the situational memory of rats by adjusting their oxidative stress levels [12]. Based on its composition and previous studies [12, 13], we hypothesized that Naosuikang participates in regulating atherosclerotic inflammation by affecting the levels of exosomal miRNAs in a way that reduces AS. In this study, we observed the effects of Naosuikang on mouse exosomal miRNAs using a mouse AS model. We found that plasma LDL, TC, and TG were reduced while HDL was increased, particularly in the medium-dose and high-dose groups, but the difference did not reach statistical significance, possibly because of an insufficient sample size. At the same time, the inflammatory factors in mice were analyzed, revealing that both the middle-dose and the high-dose groups had effective levels of CRP, TNF-α, and IL-6. In the low-dose group, this effect was not obvious. So we did not choose the low-dose group in the subsequent study. The sequence data suggest that Naosuikang can change the levels of exosomal miRNAs, given that 23 were differentially expressed in both the middle- and high-dose groups. The biological credibility analysis of differentially expressed miRNAs showed that the primary enrichment pathways of the highly expressed miRNAs were for cell division and inflammatory cytokines. Therefore, we speculated that the process by which Naosuikang reduces AS could be related to its role in reducing blood lipids and inhibiting inflammation, and exosomal miRNA could be involved in this process. The GO and KEGG analyses on the differential exosomal miRNAs implied the potential mechanism and function of Naosuikang, providing clues for further detailed research.

This study is limited by its small sample size, and this restricts the experimental results. It is also limited by the small amount of blood that was drawn from each mouse, restricting the number of laboratory tests that could be carried out, including further experimental verifications of exosomal miRNAs. Although the high dose group observed a good reduction in the value of inflammatory factors, we were very sincere because the blood samples of the high-dose group were too few to support us to complete the whole experiment. Anyway, inflammatory factors in the middle-dose group, including CRP, TNF-α and IL-6, decreased compared with the control group, probably because the sample was too small, and CRP and IL-6 did not reach statistical difference. Therefore, we believe that the use of the medium-dose group can also clarify the problem, which may not be perfect.

Conclusions

In conclusion, we speculate that exosomes are involved in the way that Naosuikang alleviates AS inflammation. Our data will facilitate explorations into the medical mechanisms of this Chinese herbal medicine.

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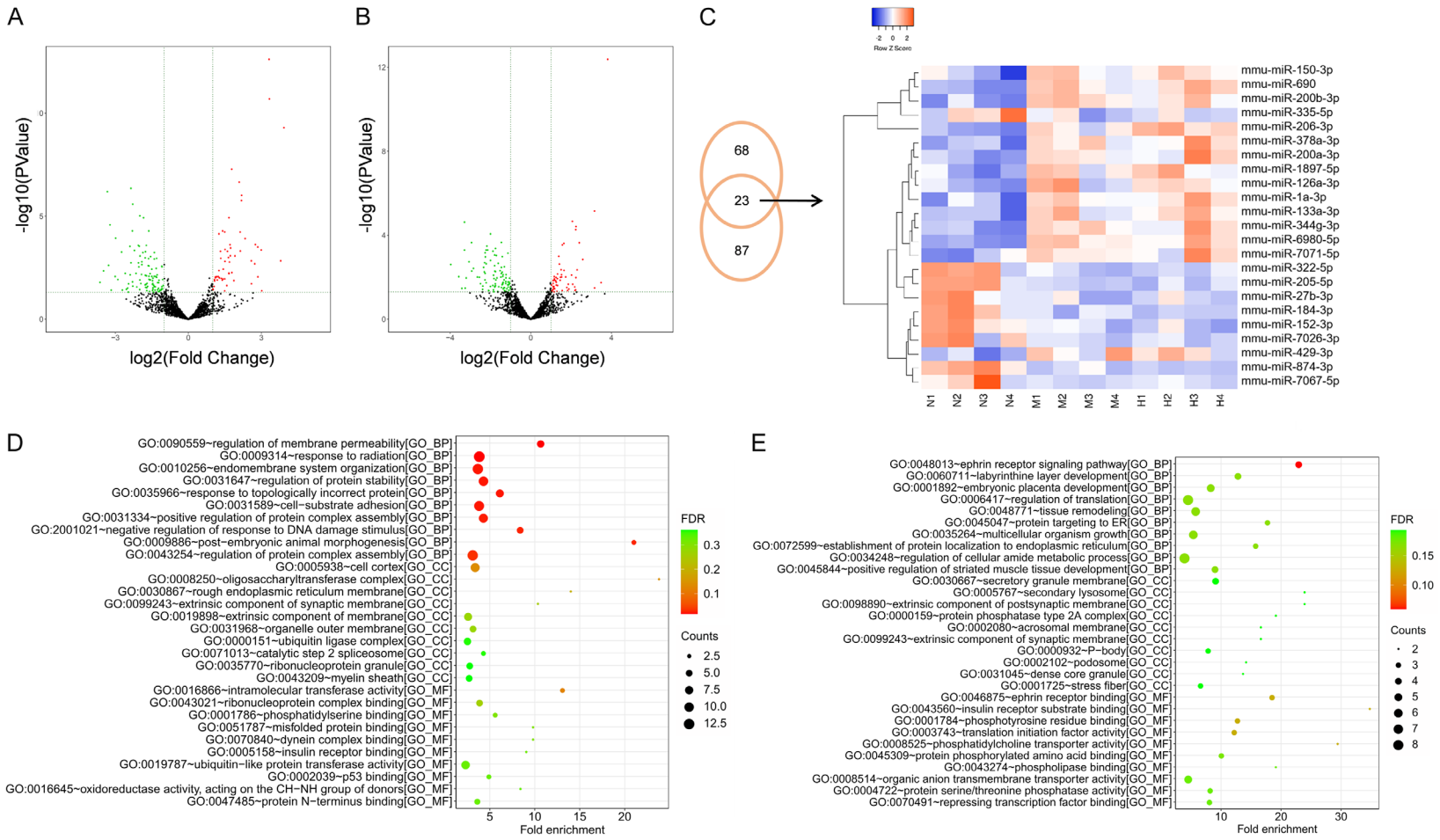
A
B
C
-log10(PValue)
log2(Fold Change)
68
23
87
mmu-miR-150-3p
mmu-miR-690
mmu-miR-200b-3p
mmu-miR-335-5p
mmu-miR-206-3p
mmu-miR-378a-3p
mmu-miR-200a-3p
mmu-miR-1897-5p
mmu-miR-126a-3p
mmu-miR-1a-3p
mmu-miR-133a-3p
mmu-miR-344g-3p
mmu-miR-6980-5p
mmu-miR-7071-5p
mmu-miR-322-5p
mmu-miR-205-5p
mmu-miR-27b-3p
mmu-miR-184-3p
mmu-miR-152-3p
mmu-miR-7026-3p
mmu-miR-429-3p
mmu-miR-874-3p
mmu-miR-7067-5p
N1 N2 N3 N4 M1 M2 M3 M4 H1 H2 H3 H4
D
GO:0090559~regulation of membrane permeability[GO_BP]
GO:0009314~response to radiation[GO_BP]
GO:0010256~endomembrane system organization[GO_BP]
GO:0031647~regulation of protein stability[GO_BP]
GO:0035966~response to topologically incorrect protein[GO_BP]
GO:0031589~cell-substrate adhesion[GO_BP]
GO:0031334~positive regulation of protein complex assembly[GO_BP]
GO:2001021~negative regulation of response to DNA damage stimulus[GO_BP]
GO:0009886~post-embryonic animal morphogenesis[GO_BP]
GO:0043254~regulation of protein complex assembly[GO_BP]
GO:0005938~cell cortex[GO_CC]
GO:0008250~oligosaccharyltransferase complex[GO_CC]
GO:0030867~rough endoplasmic reticulum membrane[GO_CC]
GO:0099243~extrinsic component of synaptic membrane[GO_CC]
GO:0019898~extrinsic component of membrane[GO_CC]
GO:0031968~organelle outer membrane[GO_CC]
GO:0000151~ubiquitin ligase complex[GO_CC]
GO:0071013~catalytic step 2 spliceosome[GO_CC]
GO:0035770~ribonucleoprotein granule[GO_CC]
GO:0043209~myelin sheath[GO_CC]
GO:0016866~intramolecular transferase activity[GO_MF]
GO:0043021~ribonucleoprotein complex binding[GO_MF]
GO:0001786~phosphatidylserine binding[GO_MF]
GO:0051787~misfolded protein binding[GO_MF]
GO:0070840~dynein complex binding[GO_MF]
GO:0005158~insulin receptor binding[GO_MF]
GO:0019787~ubiquitin-like protein transferase activity[GO_MF]
GO:0002039~p53 binding[GO_MF]
GO:0016645~oxidoreductase activity, acting on the CH-NH group of donors[GO_MF]
GO:0047485~protein N-terminus binding[GO_MF]
Fold enrichment
FDR
Counts
E
GO:0048013~ephrin receptor signaling pathway[GO_BP]
GO:0060711~labyrinthine layer development[GO_BP]
GO:0001892~embryonic placenta development[GO_BP]
GO:0006417~regulation of translation[GO_BP]
GO:0048771~tissue remodeling[GO_BP]
GO:0045047~protein targeting to ER[GO_BP]
GO:0035264~multicellular organism growth[GO_BP]
GO:0072599~establishment of protein localization to endoplasmic reticulum[GO_BP]
GO:0034248~regulation of cellular amide metabolic process[GO_BP]
GO:0045844~positive regulation of striated muscle tissue development[GO_BP]
GO:0030667~secretory granule membrane[GO_CC]
GO:0005767~secondary lysosome[GO_CC]
GO:0098890~extrinsic component of postsynaptic membrane[GO_CC]
GO:0000159~protein phosphatase type 2A complex[GO_CC]
GO:0002080~acrosomal membrane[GO_CC]
GO:0099243~extrinsic component of synaptic membrane[GO_CC]
GO:0000932~P-body[GO_CC]
GO:0002102~podosome[GO_CC]
GO:0031045~dense core granule[GO_CC]
GO:0001725~stress fiber[GO_CC]
GO:0046875~ephrin receptor binding[GO_MF]
GO:0043560~insulin receptor substrate binding[GO_MF]
GO:0001784~phosphotyrosine residue binding[GO_MF]
GO:0003743~translation initiation factor activity[GO_MF]
GO:0008525~phosphatidylcholine transporter activity[GO_MF]
GO:0045309~protein phosphorylated amino acid binding[GO_MF]
GO:0043274~phospholipase binding[GO_MF]
GO:0008514~organic anion transmembrane transporter activity[GO_MF]
GO:0004722~protein serine/threonine phosphatase activity[GO_MF]
GO:0070491~repressing transcription factor binding[GO_MF]
Fold enrichment
FDR
Counts

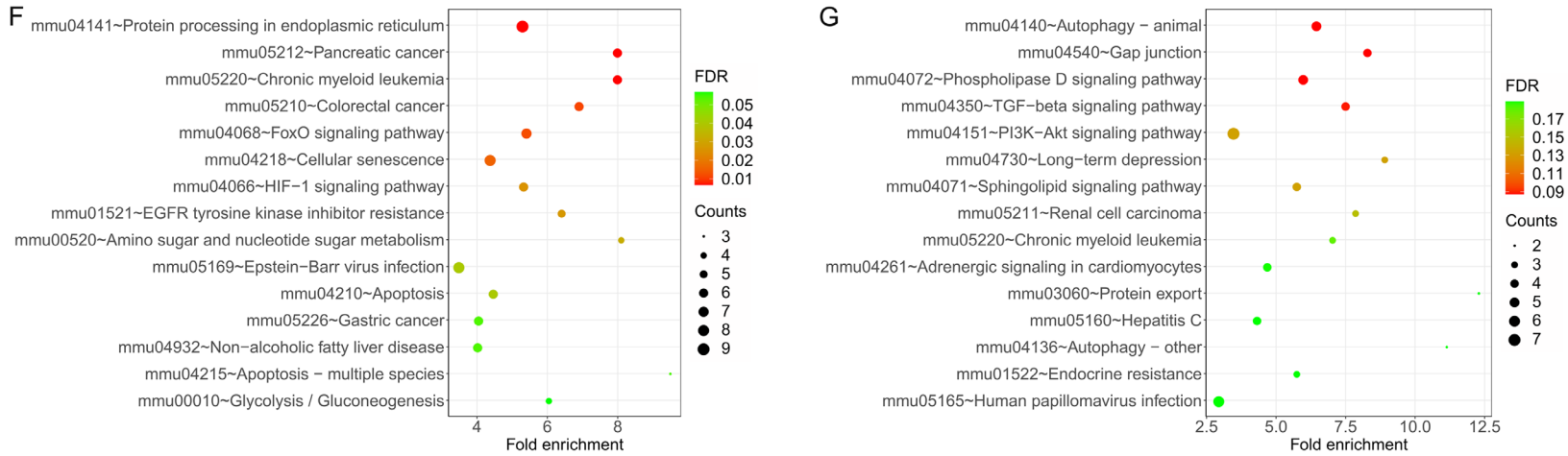

Figure 3. Analysis of miRNA sequencing results for exosomes from mice treated with Naosuikang. A. Differential exosomal miRNAs between Naosuikang middle-dose group and controls. B. Differential exosomal miRNAs between Naosuikang high-dose group and controls. C. Heatmap of differential exosomal miRNAs. D, E. Gene Ontology (GO) term analyses of candidate miRNA target genes. F, G. Kyoto Encyclopedia of Genes and Genomespathway analyses of candidate miRNA target genes.

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Disclosure of conflict of interest

None.

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