Hepatocyte-derived exosomal microRNAs orchestrate vascular inflammation and endothelial function: insights into molecular mechanisms of trimethylamine-N-Oxide in atherosclerosis

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Research

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Abstract

Background: Trimethylamine-N-oxide (TMAO) has been proved to be a new proatherogenic compound for promoting vascular inflammation and endothelial dysfunction. Hepatocyte-derived exosomes played an important role in the regulation of vascular inflammation and endothelial function. Since TMAO is produced in the liver, hepatocytes may be the first potential target of TMAO. However, it is not clear whether TMAO can directly stimulate normal hepatocytes to produce exosomes so as to mediate the motivating effects of TMAO on inflammation and endothelial dysfunction.

Methods: The hepatocytes were cultured and treated with TMAO at a physiological concentration for 24 hours (TMAO-Exos). The untreated group served as the control (Control-Exos). The exosomes were isolated from the culture supernatant and then added to the human aortic endothelial cells (HAECs) for 48 hours. The mRNA expressions of inflammatory cytokines and caspase-3 were determined by qPCR and cell apoptosis was evaluated by using the Hoechst 33342 staining solution. The miRNA profile in the exosomes were detected using an RNA-sequencing strategy. The miRNA-mRNA network was predicted, and the biological functions of the target genes were annotated by using bioinformatics methods.

Results: TMAO-Exos were able to promote the expressions of inflammatory cytokines and HAECs apoptosis. Moreover, miRNAs carried by the TMAO-Exos were quite different from that in the Control-Exos, including miR-92a-3p, miR-103-3p and miR-122-5p, etc. Further analysis showed that these differentially expressed miRNAs were predicted to target genes such as Mapk8, Casp9, Mapk10, Bcl2l11, Ikbkg and Akt1, which were supposed to be involved in the signal pathways related to vascular inflammation and endothelial function.

Conclusions: These novel results provided evidence that TMAO could indirectly talk to vascular endothelial via promoting hepatocytes to secreting exosomes that carried important genetic information, which may give a new insight into the interactions between liver and vasculature in the atherogenesis caused by TMAO. New intervention targeting this cellular crosstalk may be feasible and effective in the prevention and treatment of TMAO-induced atherogenesis.

Background

Ischemic heart disease remains a major long-term public health challenge around the world [1]. Chronic vascular inflammation and endothelial dysfunction clearly represent the characteristics of atherosclerosis [2]. In recent years, trimethylamine-N-oxide (TMAO) has been proved to be a proatherogenic compound, for exerting its pathogenic effects via promoting vascular inflammation and endothelial dysfunction [3–5]. However, the molecular mechanisms have not been completely explained.

Exosomes are nanosized membrane particles with a 50 to 100 nm size range, which are secreted by various types of cells and transmit information from cells to cells. The functions and characteristics of exosomes mainly depend on the types and states of the host cells from which they are originated. There are many kinds of proteins, lipids, microRNAs (miRNAs) and other non-coding RNAs carried by exosomes,

which were considered as the key materials for intercellular communications and play a crucial regulatory role in many biological processes such as immune response, cardiovascular disease, tumor, and neurodegenerative disease [6–8].

The latest studied indicated that hepatocyte-derived exosomes played an important role in the regulation of vascular inflammation and endothelial function [9, 10]. Since TMAO is produced in the liver [11], hepatocytes may be the first potential target of TMAO. In fact, recent research has indicated that TMAO could work directly on hepatocytes and thus exerting its influence over metabolic syndrome [12]. However, it is not clear whether TMAO can directly stimulate hepatocytes to produce exosomes and then target the vascular endothelial cells (VECs) through the circulation to mediate the motivating effects of TMAO on inflammation and endothelial dysfunction.

In the present research, TMAO was found to directly stimulate normal hepatocytes to release exosomes and thus inducing inflammation activation and cell apoptosis. Next, we utilized an RNA-sequencing strategy to characterize the miRNAs candidates contained in the exosomes, and further analysis revealed that the differentially expressed miRNAs were predicted to target the potential genes which were involved in vascular inflammation and endothelial dysfunction. These novel results provided evidence that TMAO could indirectly talk to vascular endothelial via promoting hepatocytes to secreting exosomes that carried important genetic information, which may give a new insight into the interactions between liver and vasculature in the atherogenesis caused by TMAO. Therefore, new intervention targeting this cellular crosstalk may be feasible and effective in the prevention and treatment of TMAO-induced atherogenesis.

Methods

AML12 cells culture, treatment, exosomes isolation and characterization

The AML12 cells (iCell Bioscience Inc, Shanghai) were cultured in DMEM/F12 (iCell Bioscience Inc, Shanghai) containing exosome-depleted serum (C38010050, ViVaCell, Shanghai), and treated with TMAO (T1362, Tokyo Chemical Industry Co., Ltd.) at a physiological concentration of 50 μmol/L (TMAO-Exos). The untreated group served as control (Control-Exos). After 24 hours, exosomes were isolated and purified from the culture supernatant using differential centrifugation. Briefly, the medium was collected and centrifuged at 300×g for 10min, 2000×g for 10min at 4℃ and then again at 10000×g for 30 min at 4℃. The supernatant was then passed through a 0.22-mm filter (Millipore) and ultracentrifuged at 110000×g for 70 min at 4℃. The pellets were then washed with phosphate-buffered saline (PBS) followed by a second ultracentrifugation at 110000×g for 70 min at 4℃ and then resuspended in PBS. The protein levels of the exosomes were measured using a BCA protein assay kit (23228, Thermo Scientific). The ultrastructure and size distribution of the exosomes were identified by transmission electron microscopy (JEM1200-EX, Japan) and nanoparticle tracking analysis (Nanosight NS300, Malvern, UK) respectively. Protein marker of CD81 (Servicebio, Wuhan, China) was detected by western blotting. Exosomes were labelled with DiI (Beyotime Biotechnology) for in vitro tracer experiment.

Endothelial cells culture and treatment

Human aortic endothelial cells (HAECs, iCell Bioscience Inc, Shanghai) were cultured in endothelial cell medium (ScienCell) supplemented with 5% fetal bovine serum, 1% growth factors, and 1% penicillin/streptomycin. Cells were treated with Control-Exos and TMAO-Exos at a concentration of 1: 100 (v/v) for 48 hours.

Western blot

The procedure was performed by standard protocols as previously described [13]. The exosomal protein concentration was determined using a BCA protein assay kit (23228, Thermo Scientific). Then the samples were separated by SDS-PAGE and transferred onto the Millipore polyvinylidene difluoride membranes. Primary antibody of CD81 was purchased from Servicebio, and the final antibody concentration was 1:1000. The expressions of CD81 were detected with enhanced chemiluminescence reagent (Millipore, USA).

Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR) was performed by standard protocols as previously described [14]. Briefly, total RNA was extracted from treated HAECs using TRIzol reagent (Invitrogen, USA) and concentration was measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, MA, USA). Then RNA was reverse transcribed into cDNA using the Color Reverse Transcription Kit (EZBioscience, USA). qPCR was performed on Bio-Rad CFX-96 (Bio-Rad, USA) with Color SYBR Green qPCR Master Mix (EZBioscience, USA). The mRNA expressions of interleukin-6 (IL-6), monocyte chemotactic protein-1 (MCP-1), tumor necrosis factor-α (TNF-α) and Caspase-3 were normalized to glyceraldehyde phosphate dehydrogenase (GAPDH) by using the $2^{-\triangle\triangle CT}$ method. The qPCR primers used in the study were listed in Table 1.

Cell apoptosis

Cells apoptosis was evaluated by using the Hoechst 33342 staining solution (Sigma). Briefly, the treated HAECs were washed twice with PBS and fixed with 4% paraformaldehyde for 10 minutes, and then washed twice with PBS again. Then Hoechst 33342 staining solution (10μg/mL) was added to cover cells, after incubating for 8 minutes in the dark, cells were washed twice with PBS. After that, cells were observed and imaged by a fluorescent microscope (Leica DMI4000B, Germany).

Exosomal miRNA expression profiling

Total RNA was isolated using TRIzol reagent (Invitrogen, USA), RNA concentration was measured using Qubit® RNA Assay Kit in Qubit® 2.0 Flurometer (Life Technologies, CA, USA) and RNA degradation and contamination was monitored on 1% agarose gels. After RNA quantification and qualification, 1 μg total RNA per sample was used as input material for the small RNA library. Sequencing libraries were generated using NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (NEB, USA.) following manufacturer's recommendations and index codes were added to attribute sequences to each sample

and then library quality was assessed on the Agilent Bioanalyzer 2100 system using DNA High Sensitivity Chips. After cluster generation, the library preparations were sequenced on an Illumina NovaSeq 6000 platform and 50bp single-end reads were generated. After sequencing, clean data (clean reads) were obtained by removing reads containing ploy-N, with 5' adapter contaminants, without 3' adapter or the insert tag, containing ploy A or T or G or C and low-quality reads from raw data. At the same time, Q20, Q30, and GC-content of the raw data were calculated. Then, chose a certain range of length from clean reads to do all the downstream analyses. miRNA expression levels were estimated by TPM (transcript per million) and differential expression analysis between the control-Exos and TMAO-Exos samples (three biological replicates) was performed using the DESeq (v1.22.1).

Target genes prediction and functional enrichment analysis

Potential target genes were predicted from miRDB and miRBase databases. DAVID database was used for investigating the functional annotation of the target genes. GO analysis was performed to elaborate the biological functions and KEGG pathway enrichment was used to explore the relevant signal pathways, and networks were performed on Cytoscape platform (v3.8.2) [15]. STRING database (v11.0) [16] was used for analyzing the protein-protein interactions, and networks were performed on Cytoscape platform. P-value ≤ 0.05 was considered statistically significant.

Statistical Analysis

Data were presented as mean ± standard error of the mean (SEM). Statistical analysis was conducted using the SPSS 20.0 software (SPSS Inc., Chicago, IL, USA). The comparisons between two groups were performed with independent t-test. $P < 0.05$ was considered statistically significant.

Results

Isolation and characterization of exosomes from hepatocytes culture supernatant

Nanovesicles with diameters around 100 nm were isolated and purified from the cell culture supernatant, which were consistent with the characteristic size range of exosomes under electron microscopes (Fig. 1a). The size distribution of the exosomes showed no significant difference between Control-Exos and TMAO-Exos (Fig. 1b). The expression of exosomal identity marker CD81 was determined in Control-Exos and TMAO-Exos by western blotting, protein from 293T cells was set as negative control (Fig. 1c). Exosomes were labelled with DiI and co-cultured with HAECs for 24 hours, and it was shown that DiI-labeled exosomes were internalized into HAECs (Fig. 1d).

TMAO-Exos promoted inflammatory gene expression

Control-Exos and TMAO-Exos were added to HAECs and co-cultured for 48 hours. TMAO-Exos, but not control-Exos, significantly promoted the mRNA expressions of IL-6, MCP-1 and TNF-α (Fig. 2a, b, c). The results showed that TMAO-Exos could promote inflammatory activation in HAECs.

TMAO-Exos induced cell apoptosis

Control-Exos and TMAO-Exos were added to HAECs and co-cultured for 48 hours. TMAO-Exos, but not control-Exos, significantly induced cell apoptosis (Fig. 3a, b), and promoted the mRNA expression of Caspase-3 (Fig. 3c). The results showed that TMAO-Exos could lead to HAECs damage.

The expression profile of miRNAs in TMAO-Exos.

An RNA-sequencing strategy was conducted to identify the differentially expressed miRNAs between the Control-Exos and TMAO-Exos, and the miRNAs with a P-value ≤ 0.05 were visualized on a heatmap (Fig. 4). Compaired to the untreated group, a total of 17 miRNAs were changed significantly (|log2(fold change)| ≥ 1 and P-value ≤ 0.05) after exposed to TMAO, in which eight miRNAs were considered as up-regulated, and nine of them were down-regulated (Table 2). Eight miRNAs labeled with a purely numerical code represented newly predicted genes, and it is worth noting that six of them are down-regulated, although the function is not yet known.

Prediction of miRNA-mRNA network

As we known, miRNAs have vital function for negatively regulating protein expression through destabilizing or inhibiting translation of target mRNAs [17]. Therefore, we selected the known miRNAs from Table 2 for next analysis. Thirty-one candidates predicted to interact with the nine differentially expressed miRNAs were picked out and built a miRNAs-mRNA network using Cytoscape software to exhibit the complex interaction (Fig. 5).

GO and KEGG pathway enrichment analysis

GO analysis revealed that changes in the biological processes (BP) of the predicted target genes were significantly enriched in positive regulation of apoptotic signaling pathway, positive regulation of I-kappaB kinase/NF-kappaB signaling, and protein kinase B signaling, etc. The details were specified in Additional file 1. The interacting networks among these biological processes were constructed using ClueGO of Cytoscape (Fig. 6a). KEGG pathway analysis showed that the predicted target genes were strongly associated with the signal pathways that were pivotal in regulating inflammation and endothelial function, such as NF-κB signaling pathway, TNF signaling pathway and Apoptosis (Additional file 2), and the interactions among these signal pathways were constructed using ClueGO (Fig. 6b). The NF-κB signaling pathway (Fig. 7a) and Apoptosis signaling pathway (Fig. 7b) were exported from the KEGG database, the predicted target genes were highlighted in red for showing the positions in the signaling network.

Protein-protein interactions network analysis

To further explore the interactions among the predicted target genes, protein-protein interaction (PPI) networks analysis was processed using the STRING database. The comprehensive module with the highest score (0.998) included 24 nodes and 96 edges (Fig. 8a), which was further subdivided into two

gene clusters by using MCODE of Cytoscape (Fig. 8b). Moreover, CytoHubba plug-in of Cytoscape identified Mcl1, Bcl2l11, Birc2, Mapk8, Mapk10, Aifm1, Akt1, Akt3, Casp9 and Map3k5 as the top 10 hub genes involved in this module, in which 10 nodes and 38 edges were included (Fig. 8c). Since other genes often interact with each other through these hub genes, it may be the key components of signaling pathways that control vascular inflammation and endothelial function.

Discussion

In recent years, numerous studies have showed that intestinal flora and metabolites could exert regulatory effects on atherosclerosis by inhibiting or accelerating the disease process [18]. Study has confirmed that dietary choline and phosphatidylcholine could be metabolized into trimethylamine (TMA) in the intestinal microbiota, and then TMA would be absorbed into the liver, under catalysis of the flavin monooxygenase-3, TMA was further converted to TMAO [11]. Many clinical investigations have shown that high blood TMAO levels were an independent risk factor for atherosclerosis and serious cardiovascular events [11, 19, 20]. Furthermore, TMAO has been found to promote atherosclerosis via boosting inflammatory activation and impairing VECs function both in vitro and in vivo [3, 21, 22]. Results from LDLR $^{-/-}$ mice demonstrated that increased blood TMAO levels could activate the NF-κB pathway and promote the expressions of inflammatory markers, thus leading to atherosclerosis formation [3]. And elevated plasma TMAO levels in rats could inhibit endothelial nitric oxide synthase (eNOS) expression and boost the production of inflammatory cytokine and superoxide, thus resulting in senescence-related endothelial dysfunction [21]. In vitro experiments showed that TMAO induced inflammation and endothelial dysfunction by inhibiting the activity of eNOS and provoked oxidative stress and activated inflammasome [22]. However, the effective concentrations of TMAO on VECs in vitro were much higher than the actual levels in body [3, 4, 19, 20, 22]. On the one hand, it may attribute to the complexity of the internal environment. But on the other hand, it may suggest that the molecular mechanism of TMAO on VECs remain incompletely understood, and the indirect factors contributing to inflammation and VECs injury might not be excluded. Since TMAO is produced in the live, hepatocytes may be the first potential target of TMAO. The results from recent related studies support the idea that there existed some relationships between TMAO and liver. Sifan Chen and colleagues recently found that TMAO at a physiological concentration could directly bind to hepatic endoplasmic reticulum stress kinase PERK and activated the unfolded protein response, and thus promoting metabolic dysfunction [12]. Another study in animal models of atherosclerosis has found that hepatic miR-146a-5p expression was associated with blood TMAO [23].

Recent studies have shown that exosomes, including hepatocyte-derived exosomes, were involved in atherosclerosis via regulating inflammation and vascular endothelial function [9, 24]. In addition, Hirsova P and colleagues have found that primary hepatocytes and Huh7 cells stimulated with lipid released more extracellular vesicles (which possessed the characteristic of exosomes), thereby enhancing the expressions of IL-1β and IL-6 in macrophages via the tumor necrosis factor-related apoptosis-inducing ligand contained in these vesicles [10]. In the present study, we provided evidence that TMAO at a

physiological concentration could directly stimulate normal hepatocytes to release exosomes, which were able to promote the expressions of IL-6, MCP-1 and TNF-α, and induced VECs apoptosis. As is well-known, IL-6, MCP-1 and TNF-α are the key inflammatory factors and play pivotal roles in regulation of inflammation, endothelial dysfunction, and atherosclerosis. IL-6 has been confirmed to exert central roles in the pathogenesis of atherosclerosis [25]. In hypertensive patients combined with coronary artery disease, blood MCP-1 levels were elevated and related to the degree of endothelial damage [26], and MCP-1 secretion from VECs was involved in atherosclerosis [27]. And the role of TNF-α in inducing endothelial dysfunction has been established [28]. VECs apoptosis reflects the damage of endothelial barrier and represents the initial event of atherogenesis [29]. Now widely accepted that VECs injury plays a key role in the development of atherosclerosis [30]. Our previous study has found that proinflammatory lipid could impair eNOS activity and induce VECs apoptosis [13].

It has been recognized that miRNAs carried by exosomes could be actively absorbed by both neighboring and distal cells to exert regulatory roles in the pathogenesis of cardiovascular diseases, and the specific functions depend on the host-cell molecular architecture. Besides, the differential expression of exosomal miRNAs have been found to be promising biomarkers for early detection of cardiovascular diseases [31]. The latest research showed that steatotic hepatocyte-derived exosomes could transfer miR-1 to VECs, thus inhibiting KLF4 expression and activating NF-κB pathway, thus facilitating endothelial inflammation and atherogenesis [9]. Zheng B and colleagues also found that exosomes secreted from vascular smooth muscle cells (VSMCs) mediated the communication between VSMCs and VECs by transferring KLF5-induced miR-155 to VECs, ultimately impaired endothelial function and accelerated atherogenesis progression [32]. In our study, miRNAs carried by the TMAO-treated hepatocyte-derived exosomes were quite different from that in the untreated group, including several well-known miRNAs, such as miR-92a-3p, miR-103-3p, miR-122-5p and miR-199a-3p, etc. Further bioinformatics analysis showed that these differentially expressed miRNAs were predicted to target mRNAs such as Mapk8, Casp9, Mapk10, Bcl2l11, Ikbkg and Akt1, which were supposed to be involved in the signal pathways related to inflammation and vascular endothelial function, including cell apoptosis. A large body of evidence has demonstrated that NF-κB signaling pathway, TNF signaling pathway, Apoptosis signaling pathway, Toll-like receptor signaling pathway and FoxO signaling pathway play a crucial role in inflammation, endothelial function, and atherosclerosis [28, 29, 33–36]. Particularly, NF-κB signaling pathway has been at the crossroads of inflammation and atherogenesis [33], and specific NF-κB inhibition of endothelial cell reduced proinflammatory gene expression such as IL-6 and TNF, and protected $ApoE^{-/-}$ mice from atherosclerosis [34]. Recent studies have revealed that exosome derived from CD137-modified endothelial cells could promote Th17 cell differentiation and atherosclerosis progression in $ApoE^{-/-}$ mice through NF-κB pathway mediated IL-6 expression [37], and exosomes released from mature dendritic cells carried TNF-a on exosome membrane, which was found to be efficient in activating the NF-κB pathway, thus provoking endothelial inflammation and atherosclerosis [24]. Furthermore, there exists a close relationship between the NF-κB pathway and the inflammatory cytokines of IL-6, MCP-1 and TNF-α. IL-6 and MCP-1 contain the binding elements for NF-κB, which is critical for transcriptional induction of IL-6

and MCP-1 genes, and TNF-a serves as one of the NF-kB-activating factors, inducing IL-6 and MCP-1 transcription intensely [38, 39].

So far, to our best knowledge, no studies have defined the relationship between hepatocyte-derived exosomes and the facilitating effects of TMAO on vascular inflammation and endothelial dysfunction. The intriguing findings demonstrated that hepatocyte-derived exosomes undertook the proatherogenic roles of TMAO in the vasculature, at least in part, via transferring a cluster of miRNAs to the endothelial cells. And further analysis indicated that the level and diversity of the exosomal miRNAs could be directly correlated with the TMAO-induced vascular inflammation and endothelial dysfunction. Future investigation of the precise mechanisms whereby circulating exosomal miRNAs interact with the corresponding target genes to irritate inflammatory response and impair endothelial function will increase our knowledge of the roles of TMAO in vascular health and atherosclerosis.

Conclusions

In the present study, we found that TMAO was able to stimulate normal hepatocytes to produce exosomes, which could be absorbed by endothelial cells, thus enhancing the mRNA expressions of inflammatory cytokines and cell apoptosis via transferring a cluster of miRNAs to the VECs and regulated signal pathways related to inflammation and vascular endothelial function. These studies may provide a new insight into the interactions between liver and vascular endothelial in the atherogenic process orchestrated by TMAO, at least in part, could be attributable to the hepatocyte-derived exosomes and the loaded miRNAs, which suggests that targeting this cellular crosstalk may provide a novel approach that can restrain the deleterious effects induced by TMAO on VECs.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

XL, JT and JMC conceived and designed the study as well as analyzed and interpreted the data; JT and JMC directed the research and revised the manuscript; XL and YJS drafted the manuscript; XL, YJS, JZCT and JPS performed the experiments. LFL helped with analysis and interpretation of some data. All authors read and approved the final manuscript.

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Abbreviations

TMAO: trimethylamine-N-oxide; miRNAs: microRNAs; VECs: vascular endothelial cells; AML12: alpha mouse liver 12; DMEM/F12: Dulbecco's Modified Eagle Medium/Nutrient Mixture F-12; Control-Exos: exosomes isolated from TMAO-untreated group; TMAO-Exos: exosomes isolated from TMAO-treated group; PBS: Phosphate-buffered saline; HAECs: human aortic endothelial cells; BCA: bicinchoninic acid; SDS-PAGE: sodium dodecyl sulfate polyacrylamide gel electrophoresis; qPCR: quantitative polymerase chain reaction; cDNA: complementary DNA; IL-6: interleukin-6; MCP-1: monocyte chemotactic protein-1; TNF-α: tumor necrosis factor-α; GAPDH: glyceraldehyde phosphate dehydrogenase; DAVID: the database for annotation, visualization and integrated discovery; GO: Gene ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; SEM: standard error of the mean; 293T: human embryonic kidney 293T; BP: biological processes; NF-κB: nuclear factor-κB; PPI: protein-protein interaction; Mcl1: MCL1 apoptosis

regulator, BCL2 family member; Bcl2l11: BCL2 apoptosis regulator like 11; Birc2: baculoviral IAP repeat containing 2; Mapk8: mitogen-activated protein kinase 8; Mapk10: mitogen-activated protein kinase 10; Aifm1: apoptosis inducing factor mitochondria associated 1; Akt1: AKT serine/threonine kinase 1; Akt3: AKT serine/threonine kinase 3; Casp9: caspase 9; Map3k5: mitogen-activated protein kinase kinase kinase 5; TMA: trimethylamine; LDLR: low density lipoprotein receptor; eNOS: endothelial nitric oxide synthase; PERK: eukaryotic translation initiation factor 2 alpha kinase 3; IL-1β: interleukin-1β; KLF4: Kruppel like factor 4; VSMCs: vascular smooth muscle cells; KLF5: Kruppel like factor 5; Ikbkg: inhibitor of nuclear factor kappa B kinase regulatory subunit gamma; FoxO: forkhead box, sub-group O; ApoE: apolipoprotein E.

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Tables

Table 1 Quantitative polymerase chain reaction (qPCR) primers used in the study

Name	Species	Forward Sequence	Reverse Sequence
IL-6	Homo	TGGCAGAAAACAACCTGAACCTT	TCTGGCTTGTTCCTCACTACTCT
MCP-1	Homo	CTCATAGCAGCCACCTTCATTCC	GATCACAGCTTCTTTGGGACACT
TNF-α	Homo	CTCAGCCTCTTCTCCTTCCTGAT	TCGAGAAGATGATCTGACTGCCT
Caspase-3	Homo	CGGCGCTCTGGTTTTCGTTA	GTCCAGGGATATTCCAGAGTCCA
GAPDH	Homo	TGCACCACCAACTGCTTAGC	GGCATGGACTGTGGTCATGAG

Table 2 The differentially expressed miRNAs between the Control-Exos and TMAO-Exos

	ID	Control-Exos_mean	TMAO-Exos_mean	Fold Change_Log2	P_value
Up	6_9856	53136.4	114742.9	1.110617915	4.24E-36
	7_11542	44.3	660.1	3.867285559	0.042018456
	miR-103-3p	1545.3	3120.6	1.013465437	0.017122205
	miR-302a-3p	0	467	8.87036472	0.035026562
	miR-302b-3p	0	3401.8	11.73250664	8.55E-12
	miR-302d-3p	0	766	9.583082768	0.003244933
	miR-744-5p	3541.9	7435.2	1.069634827	0.000621541
	miR-92a-3p	633.3	2048.6	1.692105134	0.001897612
Down	12_19875	3430.1	1407.5	-1.28451161	0.005508287
	15_23079	2314.9	479.1	-2.270166118	8.33E-05
	18_26561	492.8	54.4	-3.155968955	0.023602876
	2_2684	513.3	37.4	-3.743431938	0.014180519
	4_5543	443.1	17.1	-4.616822969	0.032070247
	9_14879	1712.3	456.2	-1.905880482	0.001012913
	miR-122-5p	2676.9	658.7	-2.021220078	0.000116753
	miR-199a-3p	672.1	0	-9.394677046	0.000582822
	miR-199b-3p	529	0	-9.049848549	0.003405104

Figures

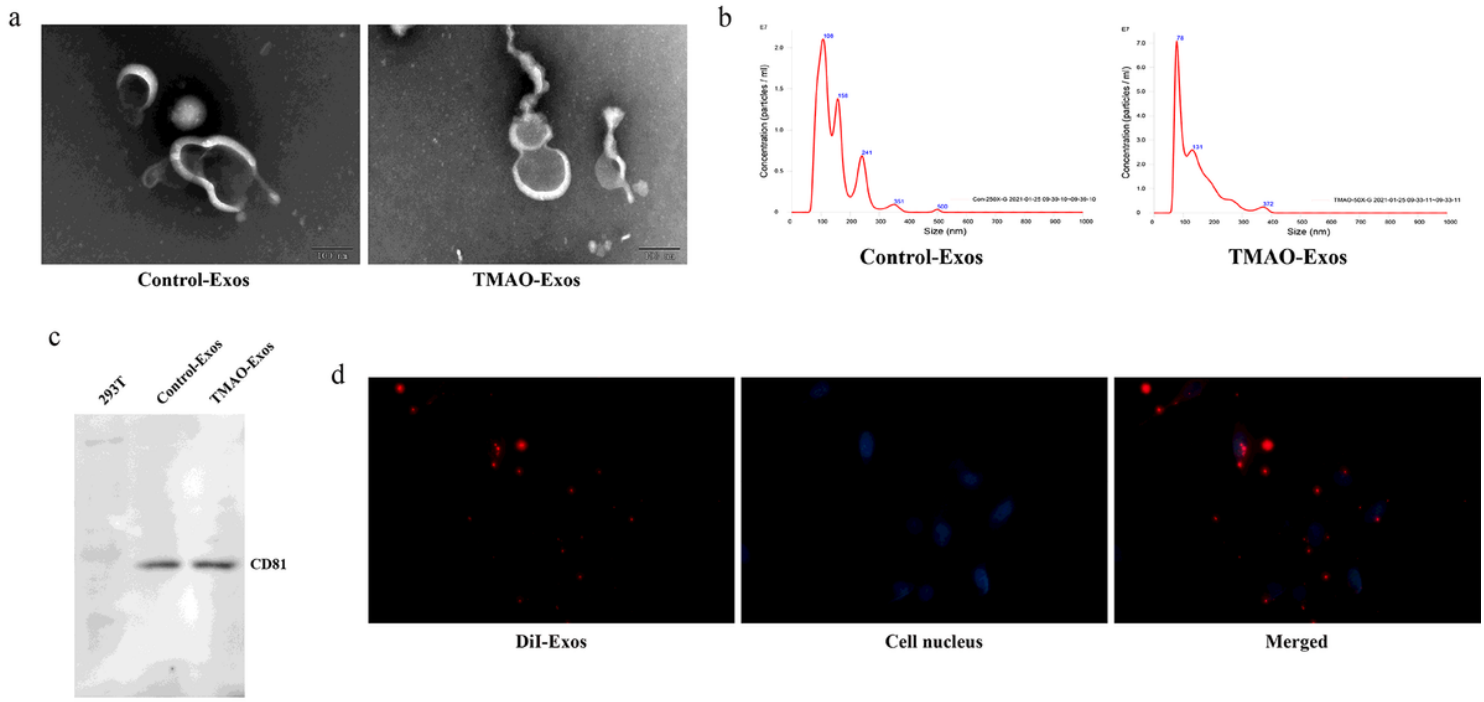

Figure 1

Isolation and characterization of exosomes from hepatocytes culture supernatant. Nanovesicles with diameters around 100 nm were isolated and purified from the AML12 cells culture supernatant, which were consistent with the characteristic size range of exosomes under electron microscopes (a). The size distribution of the exosomes showed no significant difference between Control-Exos and TMAO-Exos (b). The expression of exosomal identity marker CD81 was determined in Control-Exos and TMAO-Exos by western blotting, protein from 293T cells was set as negative control (c). Exosomes were labelled with DiI and co-cultured with HAECs for 24 hours, it was shown that DiI-labeled exosomes were internalized into HAECs (d).

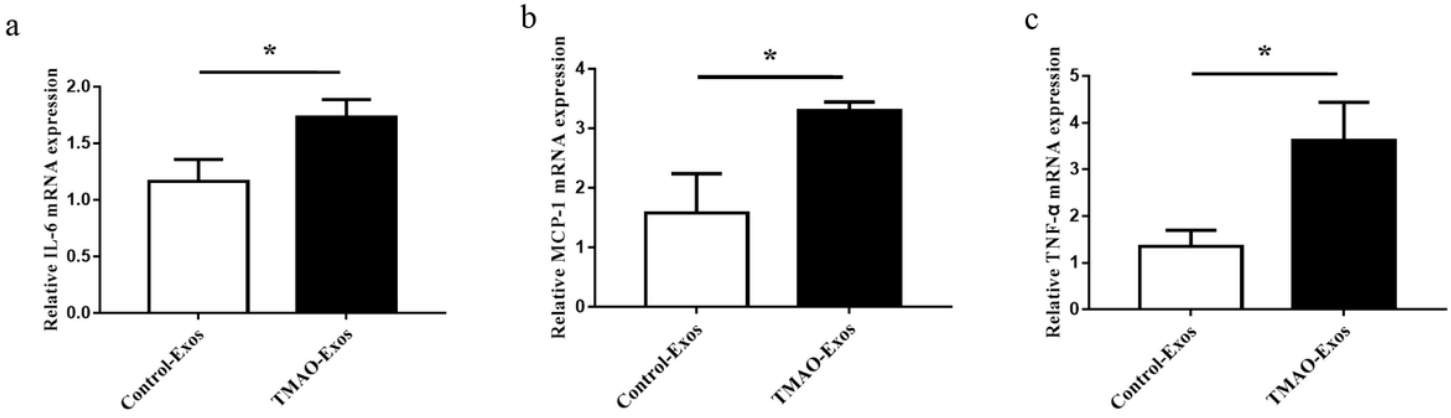

Figure 2

TMAO-Exos promoted inflammatory gene expression. Control-Exos and TMAO-Exos were added to HAECs and treated for 48 hours. Compared with Control-Exos, TMAO-Exos significantly promoted the

expressions of IL-6 (a), MCP-1v(b) and TNF-α (c). All data were expressed as mean±SEM. qPCR genes normalized to GAPDH expression. n=3, independent t-test was performed for comparisons; *P<0.05.

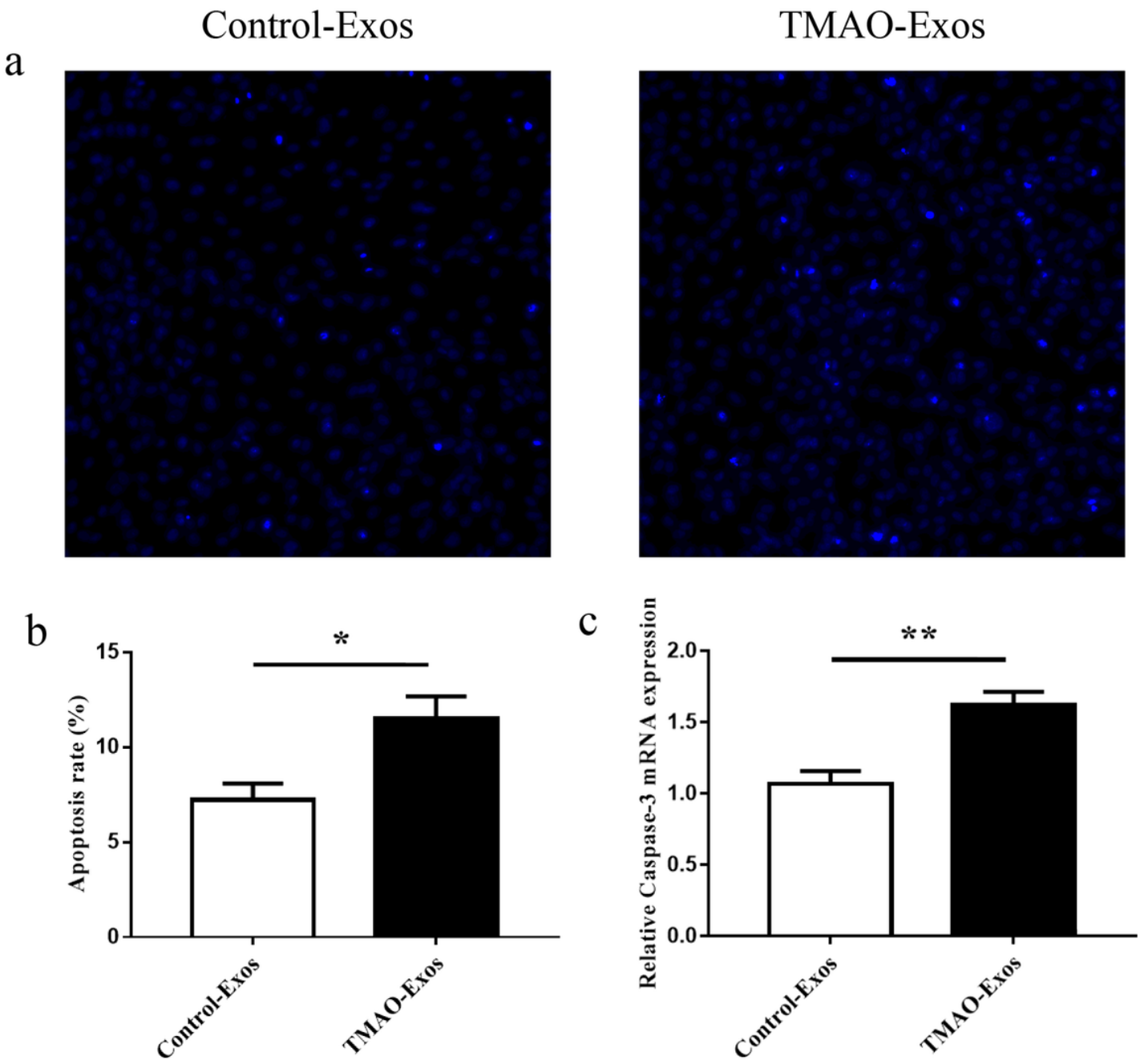

Figure 3

TMAO-Exos induced cell apoptosis. Control-Exos and TMAO-Exos were added to HAECs and co-cultured for 48 hours. TMAO-Exos, but not control-Exos, significantly induced cell apoptosis (a, b), and promoted the mRNA expressions of Caspase-3 (c). All data were expressed as mean±SEM. qPCR genes normalized to GAPDH expression. n=3, independent t-test was performed for comparisons; *P<0.05; **P<0.01.

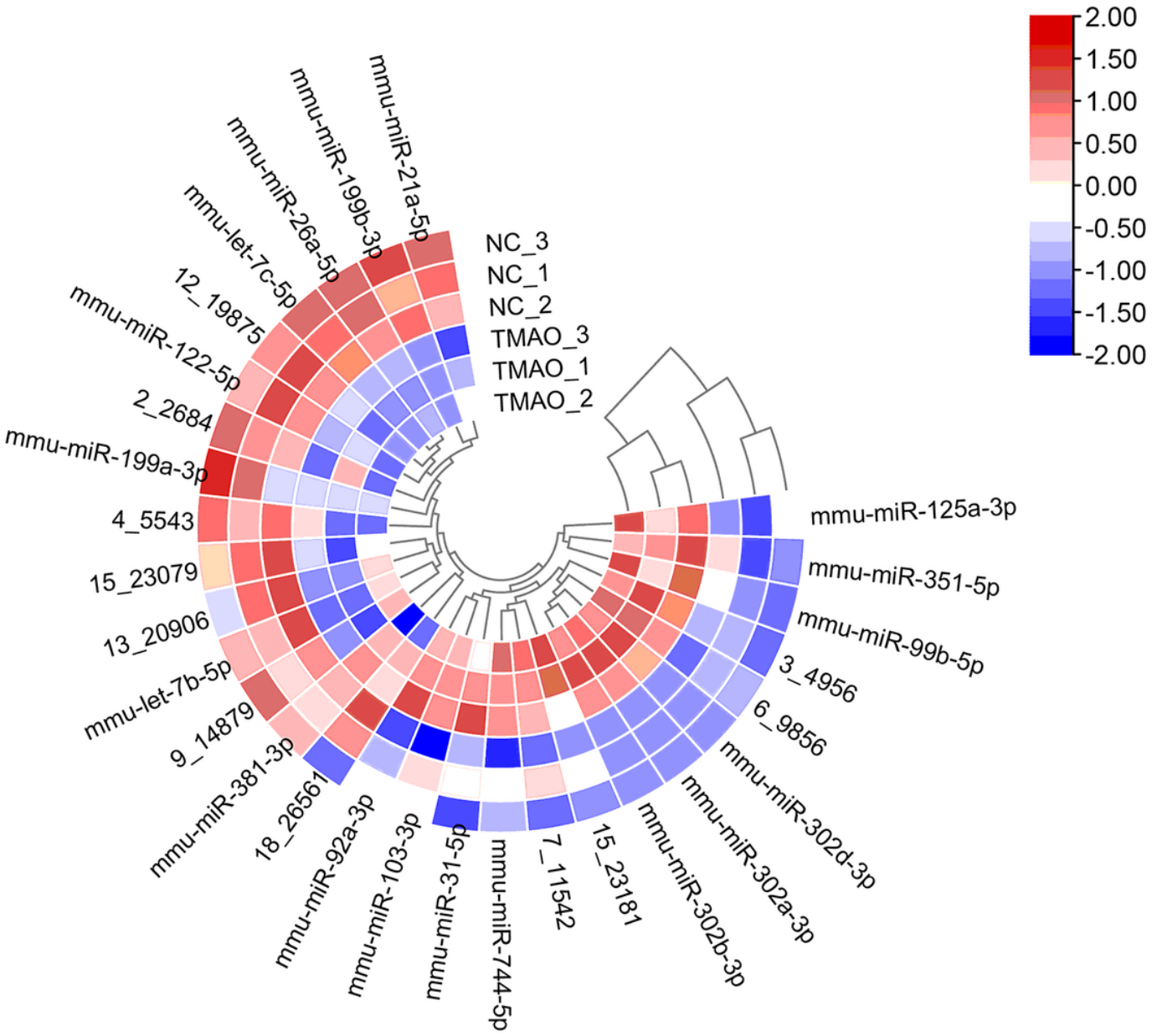

Figure 4

The expression profile of miRNAs in TMAO-Exos. An RNA-sequencing strategy was conducted to identify the differentially expressed miRNAs between the Control-Exos and TMAO-Exos, and the miRNAs with a P-value ≤ 0.05 were visualized on a heatmap.

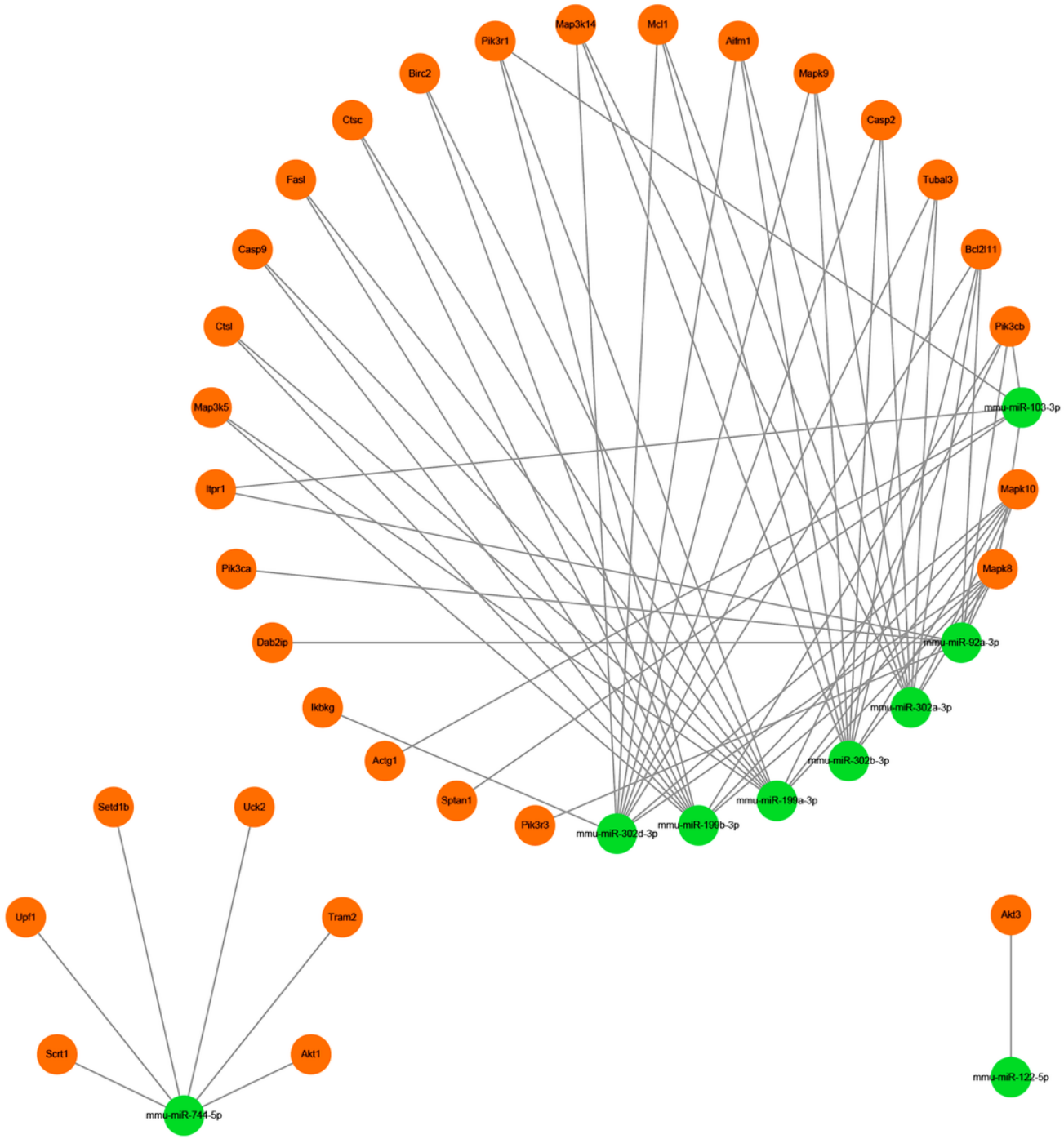

Figure 5

Prediction of miRNA-mRNA network. The known miRNAs were selected from the Table 2 for next analysis. Thirty-one candidates predicted to interact with the nine differentially expressed miRNAs were picked out and built a miRNAs-mRNA network using Cytoscape software to exhibit the complex interaction.

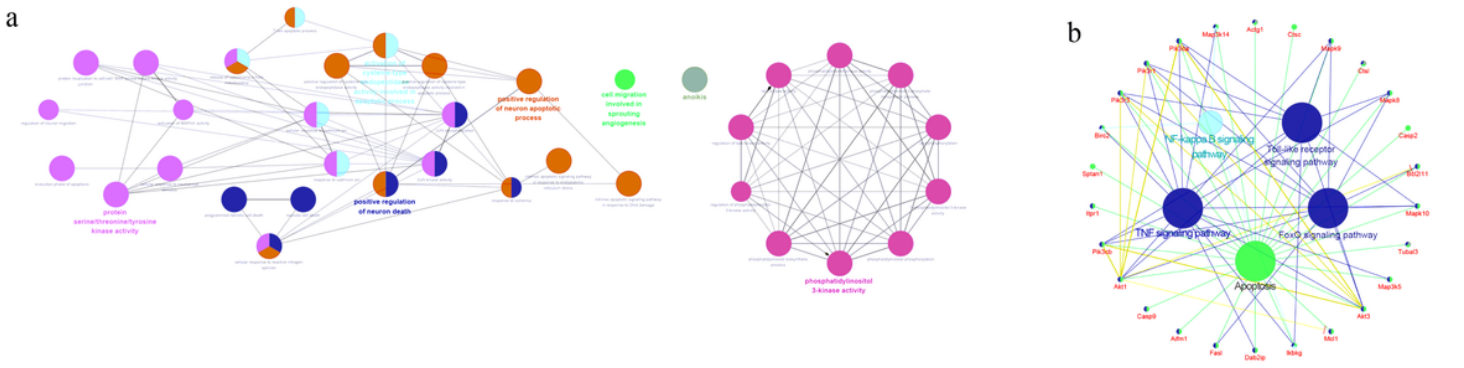

Figure 6

GO and KEGG pathway enrichment analysis. GO analysis revealed that changes in the biological processes (BP) of the predicted target genes were significantly enriched in positive regulation of apoptotic signaling pathway, positive regulation of I-kappaB kinase/NF-kappaB signaling and phosphatidylinositol-mediated signaling and protein kinase B signaling, and the interacting networks among these biological processes were constructed using ClueGO of Cytoscape (a). KEGG pathway analysis showed that the predicted target genes were strongly enriched in NF-κB signaling pathway, TNF signaling pathway and Apoptosis, and the interactions among these signal pathways were constructed using ClueGO (b).

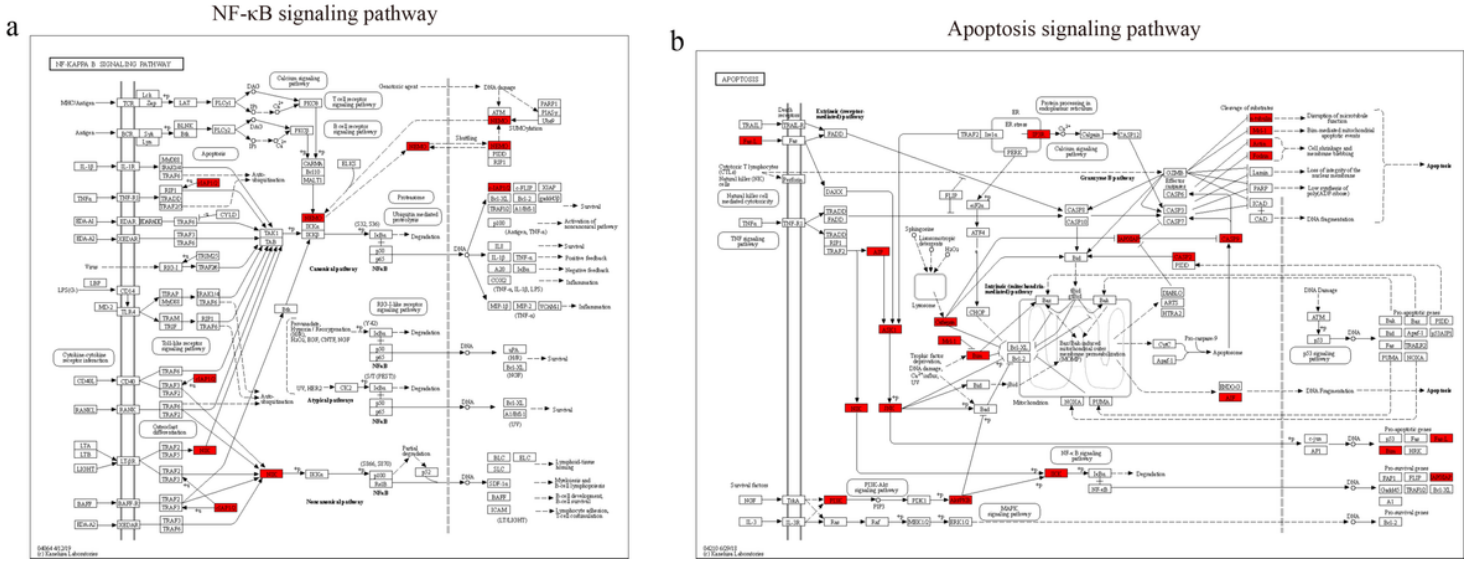

Figure 7

The positions of the predicted target genes in the signaling network. The NF-κB signaling pathway (a) and Apoptosis signaling pathway (b) were exported from KEGG database, the predicted target genes were highlighted in red for showing the positions in the signaling network.

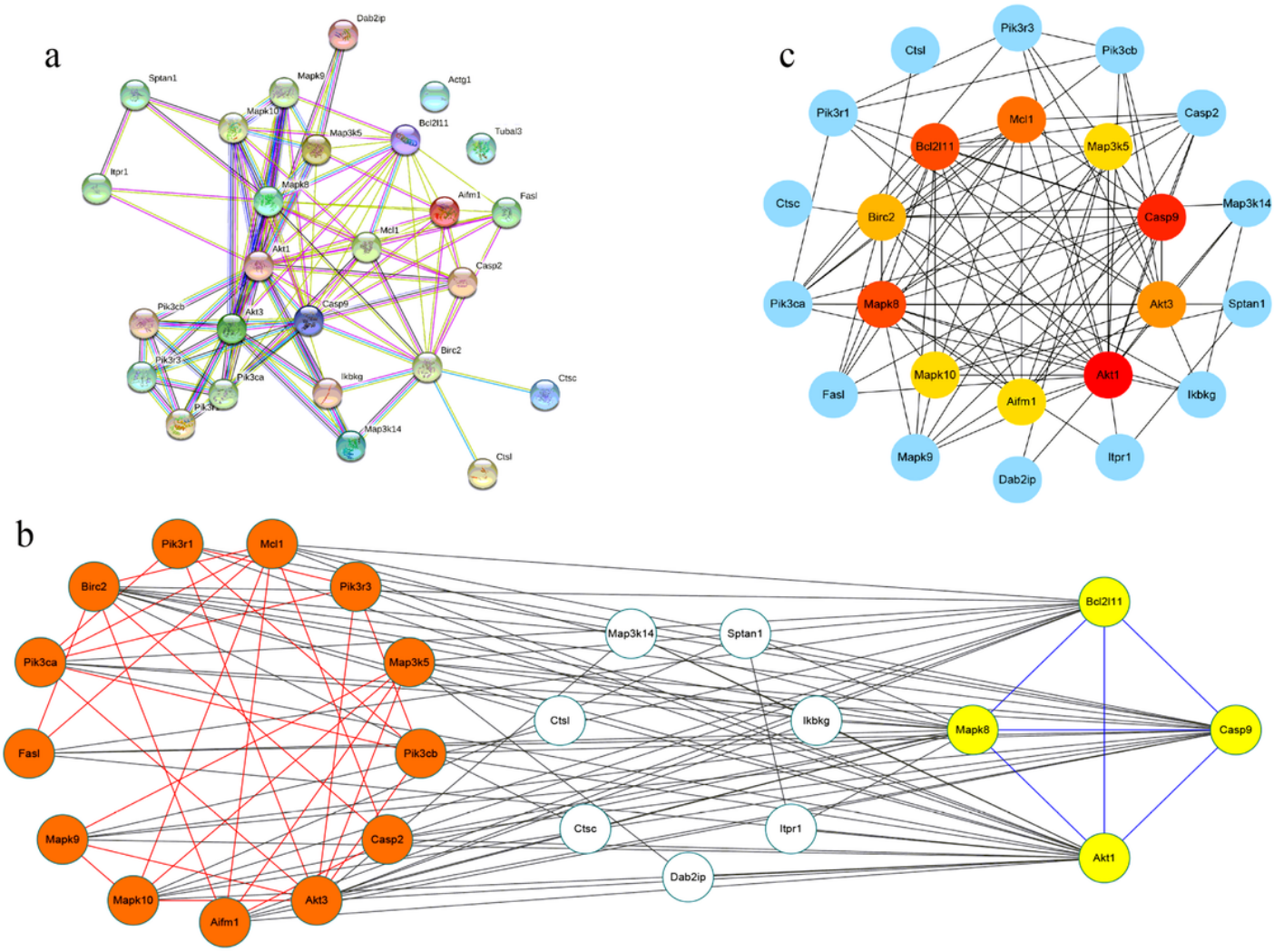

Figure 8

Protein-protein interactions network analysis. Protein-protein interaction (PPI) networks analysis was processed using the STRING database. The comprehensive module with the highest score (0.998) included 24 nodes and 96 edges (a), which were further subdivided into two gene clusters by using MCODE of Cytoscape (b). Moreover, CytoHubba plug-in of Cytoscape identified Mcl1, Bcl2l11, Birc2, Mapk8, Mapk10, Aifm1, Akt1, Akt3, Casp9 and Map3k5 as the top 10 hub genes involved in this module, in which 10 nodes and 38 edges were included (c).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Additionalfile1.TableS1..xlsx
- Additionalfile2.TableS2..xlsx