Hepatocyte-Derived Extracellular Vesicles Promote Endothelial Inflammation and Atherogenesis via microRNA-1

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ABSTRACT

**Background and Aims:** Clinical evidence has indicated a close relationship between non-alcoholic fatty liver disease (NAFLD) and cardiovascular diseases (CVD). However, the underlying mechanism remains to be elucidated. This study aimed to explore a potential role of hepatocyte-derived extracellular vesicles (EVs) in endothelial inflammation and atherogenesis under NAFLD status.

**Methods and Results:** By using an endothelial cell-specific PCR array, we identified that hepatocyte-derived EVs from the fatty liver are a potent inducer of endothelial inflammation. Through profiling and functional analysis of the global microRNA in the EVs, we found that microRNA-1 (miR-1) is a key EV cargo that mediates the pro-inflammatory effect of EVs by down-regulation of KLF4 and activation of the NF-κB pathway in endothelial cells. Moreover, not only did the inhibition of miR-1 reduce endothelial inflammation *in vitro* but also attenuated atherogenesis in ApoE-deficient mice.

**Conclusion:** Steatotic hepatocyte-derived EVs promote endothelial inflammation and facilitate atherogenesis by miR-1 delivery, KLF4 suppression and the NF-κB pathway activation. The findings illustrate an important role for hepatocyte-derived EVs in distant communications between the liver and vasculature, suggesting a new mechanism underlying the link between NAFLD and CVD.
LAY SUMMARY

Non-alcoholic fatty liver disease (NAFLD), a condition highly prevalent in obese or diabetic patients, is emerging at a high risk for the development of cardiovascular diseases (CVD). In the current study, we identified a kind of toxic particles, namely extracellular vesicles, released by hepatocytes under NAFLD conditions, which causes vascular endothelial inflammation and promotes atherosclerosis. Furthermore, we identified a small molecular cargo in the toxic particles, which is a potent inducer of endothelial inflammation. By inhibiting this cargo function, a specific gene-based inhibitor profoundly attenuates atherogenesis in mice. Thus, our study uncovers a novel mechanism for distant communications between the liver and blood vessels and suggests a new way to prevention and treatment of CVD, especially for NAFLD patients.

HIGHLIGHTS:

- Extracellular vesicles (EVs) derived from steatotic hepatocytes are potent inducers of endothelial inflammation.
- MicroRNA-1 (miR-1) is a key molecular cargo of EVs responsible for the pro-inflammatory effect of steatotic hepatocytes.
- The effect of miR-1 on endothelial cells is mediated by KLF4 suppression and NF-κB activation.
- Inhibition of miR-1 profoundly reduces endothelial inflammation and attenuates atherogenesis.
- Hepatocyte-derived EVs plays a key role in distant communications between the liver and vasculature.
Nonstandard Abbreviations and Acronyms:

3’-UTR, 3’-untranslated region
CVD, cardiovascular diseases
EV, extracellular vesicle
FFA, free fatty acid
HFD, high fat diet
HUVEC, human umbilical vein endothelial cells
ICAM-1, intracellular adhesion molecule-1
KLF4, Kruepple-like factor 4
miRNA, microRNA
miR-1, microRNA-1
NAFLD, non-alcoholic fatty liver disease
NTA, nanoparticle tracking analysis
PA, palmitic acid
PMH, primary murine hepatocytes
qPCR, quantitative RT-PCR
SMA, smooth muscle actin
VCAM-1, vascular cell adhesion molecule-1
INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) has become a major public health concern worldwide. The disease currently affects 20-35% of the general population in Western countries, and 10% of patients can progress from benign steatosis to more severe conditions, including steatohepatitis, cirrhosis, and liver failure. Moreover, multiple lines of evidence have revealed a strong association between NAFLD and several markers of subclinical atherosclerosis independent of traditional risk factors. Thus, not only is the adverse effect of NAFLD confined to the progression of deteriorated liver injury, but also confers an independent risk for the development of atherosclerosis and other related cardiovascular diseases (CVD).

Atherosclerosis is regarded as a chronic inflammatory metabolic disease caused by aberrant accumulation of lipids and infiltration of inflammatory cells at the vascular endothelium. It is believed that the first step in the initiation of atherosclerosis is endothelial injury and dysfunction. Both clinical evidence and experimental data have demonstrated a close link between NAFLD, endothelium dysfunction and CVD, whereas the underlying mechanism remains to be elucidated.

The link between NAFLD and CVD indicates the importance of intercellular communication in the pathogenic process of the diseases. It is now well recognized that cells communicate not only via direct contact and soluble factors, but also by membrane-derived nanometer-sized vesicles, namely, extracellular vesicles (EVs). EVs carry numerous donor cell-derived molecules, including proteins, lipids and nucleic acids. By transferring these bioactive contents into target cells, EVs play essential roles in intercellular communications. The EVs are released under either physiologic or pathologic conditions, including liver diseases, and exert a wide range of effects on target cells. Among the various molecular cargos, microRNAs (miRNAs) are regarded as essential to the function of EVs.
The highly conserved, noncoding small RNAs consisting of 19–26 nucleotides, are key regulators of gene expression in a wide range of organisms. They usually contain a 6- to 8-nucleotide seed sequence, corresponding to complementary sequences in the 3’-untranslated region (3’-UTR) of target mRNAs. Thus, miRNAs can suppress protein expression of targeted mRNAs through either translational arrest or mRNA degradation. Serving as an important cargo of EVs, miRNAs have recently emerged as key regulators involved in the pathogenesis of various diseases, including NAFLD and CVD. In the present study, we provide the first evidence that EVs released from steatotic hepatocytes are potent inducers of endothelial inflammation. Furthermore, we identified miR-1 as a key molecule mediating the pro-inflammatory effect of EVs via down-regulation of KLF4 and activation of the NF-κB pathway in endothelial cells. Moreover, inhibiting miR-1 with a specific antagonir profoundly attenuated atherogenesis in ApoE-deficient mice. Thus, our findings uncover an important role of hepatocyte-derived EVs in endothelial inflammation and suggest a novel pathogenic mechanism underlying the development of CVD associated with NAFLD.
METHODS

Cell Culture, transfection, and treatments

All cell lines were maintained at 37°C with 5% CO2 in Dulbecco`s Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Primary murine hepatocytes were isolated by using a collagenase perfusion method and purified by Percoll gradient centrifugation as described previously. Viable hepatocytes were determined by trypan blue and cultured for the experiments. Huh7 hepatocyte line was purchased from JCRB (Japan), and HEK293 and THP-1 cell lines from ATCC (USA). Human umbilical vein endothelial cells (HUVECs) were isolated from donated umbilical cords as previously described. HUVECs used in the study were passage 2-6 and cultured on collagenase-coated dishes with endothelium cell basal medium (ScienCell Research Laboratories, San Diego, USA) containing growth factors, 10% FBS, streptomycin (100 IU/mL), penicillin (100 IU/mL) and Amphotericin B (10 μg/L). FuGene HD (Roche) and Lipo 3000 reagents (Gibco) were used for transfections. Palmitic acid (PA) was dissolved in 0.1 M NaOH by heating at 90°C for 10 min, and then a stock solution was prepared containing 5 mM free fatty acid (FFA) coupled with 5% (w/v) fatty acid-free BSA (Sigma, St. Louis, USA). For treatment of cells, the stock FFA solution was added into serum-free medium at the indicated concentrations for the desired time period.

Isolation and characterization of hepatocyte-derived extracellular vesicles (EVs)

EVs were isolated from cell culture media of primary murine hepatocytes or Huh7 cell line by differential centrifugation as previously described. Briefly, hepatocytes were grown to 90% confluence in 6x100mm tissue-culture dishes and treated with 200μM PA or vehicles alone in serum-free medium (10ml/dish) for 18 hrs. After treating and removing cells and other debris by centrifugation, the media was differential centrifuged at 2000 xg and 10,000 xg, respectively, for 30 minutes.
The supernatant was then filtered (0.22 μm filter) and re-centrifuged at 120,000 x g for 2 hrs at 4°C. The pellets containing EVs were re-suspended in PBS and centrifuged at 120,000 x g for 2 hrs again. Each resultant pellet was finally re-suspended in PBS buffer for use or stored at 4°C for less than 3 days for further experiments. The size and number of EVs were determined by nanoparticle tracking analysis (NTA) with Nanosight NS 300 (Malvern Panalytical, UK). The EVs were identified by transmission electronic microscope analysis at Fudan University Electron Microscopy Center. Briefly, EVs were fixed in 1.5 M sodium cacodylate buffer (pH 7.4), absorbed onto formvar-coated copper grids (Science Services, München), and examined by electronic microscope (Tecnai G2 Spirit, FEI Corp., USA). Images were taken with an AMT digital camera for data acquisition.

**Western blot analysis**

Western blot analysis was performed according to the standard protocol as previously described \(^2\)\(^1\). The details of antibodies used in this study are shown in the Supplementary CTAT Table.

**RNA extraction, small RNA-sequencing and quantitative real-time PCR**

Mouse primary hepatocytes were isolated from DIO or control mice, and hepatocyte-derived EVs were isolated from conditioned medium. Total RNAs in the EVs were extracted using the SeraMir exosome RNA kit (SBI, Mountain View, USA) according to the manufacturer's instructions. RNA-seq was conducted on an Illumina HiSeq 2500 at Guangzhou RiboBio (Guangzhou, China). Briefly, RNAs were ligated with 3’RNA adapter, followed by 5’adapter ligation. Subsequently, the adapter-ligated RNAs were subjected to RT-PCR and amplified with a low-cycle. Then the PCR products were size selected by PAGE gel according to instructions of NEBNext® Multiplex Small RNA Library Prep Set for Illumina® (Illumina, USA). The purified library products were evaluated using the Agilent 2200 TapeStation and
diluted to 10 pM for cluster generation in situ on the HiSeq2500 single-end flow cell followed by sequencing (1x50 bp) on HiSeq 2500. The quality of the sequencing data was analyzed by the bioinformatics team associated with the sequencing core. Quantitative real-time quantitative PCR (qPCR) was performed with an Applied Biosystems 7500 using SYBR Premix EX Taq (Takara, Tokyo, Japan). In brief, the RNA was reverse transcribed to cDNA using small RNA assays and miRNA-specific primers (RiboBio), according to the manufacturer's instructions. miR-16 was included as an internal standard. Each sample was analyzed in triplicate in an RT-PCR assay, and the mean threshold cycle (Ct) value of the triplicates was used in the final data analysis. The qPCR results were first normalized to the Ct value of miR-16, referred to as ΔCt. The relative miRNA expression levels were calculated using the 2^−ΔΔCt method.

**EV internalization assay**

Hepatocyte-derived EVs were fluorescently labeled with the cell membrane marker, PKH26 (Sigma, USA), prior to incubation with HUVECs. After 12-hrs incubation, HUVECs were washed vigorously, fixed by 4% paraformaldehyde and counterstained with DAPI. Control cells were treated with PBS vehicle alone. Microscopic images were analyzed using Image J.

**Co-culture experiments**

Co-culture experiments were conducted in 6-well Transwell plates with 0.4 µm pore-sized filters (Corning Costar, USA). Huh7 cells were seeded in each transwell insert with HUVECs seeded at the bottom chamber. Both hepatocytes and HUVECs were washed with PBS before starting the co-culture experiments.

**Human Endothelial Cell Biology PCR Array**
HUVECs were treated for 24 hrs with the EVs isolated from PA-treated Huh7 hepatocytes (PAEV) or the control cells (CtlEV), total RNA was then extracted from the treated endothelial cells using an RNeasy Mini Kit (Qiagen; Carlsbad, USA) with DNase digestion. 1.0 μg of total RNA was reverse transcribed using a QuantiTect reverse transcription kit (Qiagen). The cDNA products were analyzed using a Human Endothelial Cell Biology RT2 Profiler™ PCR array (Qiagen) according to the manufacturer's instructions. The data were further validated by qPCR with the QuantiTect SYBR green PCR kit (Qiagen) and analyzed through the vendor’s web-based module (ΔΔCq method) as previously described.

**Adhesion Assay**

Adherence of monocytes to endothelial cells was analyzed as described previously. In brief, THP-1 cells were labeled with a fluorescent probe, BCECF-AM (APExBIO, Huston, USA). After washing, the labeled monocytes (1x10⁴ cells/well) were added to the monolayers of HUVECs treated with or without TNF-α (1 ng/ml) for 6h. Nonadherent cells were removed by rinsing the plates 3 times, and the number of adherent cells counted under a fluorescence microscope.

**Transfection of the miR-1 inhibitor or mimic**

For the functional analysis of miR-1, a commercially available miR-1 inhibitor or mimic and a negative control inhibitor or mimic (mirVana™; Invitrogen, Thermo Fisher Scientific, Waltham, USA) were used. The transfection was conducted using the complexes of Lipofectamine 3000 (Invitrogen) and miR-1 inhibitor/mimic (50nM) or negative controls according to the manufacturer’s instructions.

**Adenovirus infection and siRNA knockdown of KLF4**

For overexpressing KLF4, HUVECs were infected with control or KLF4 expression adenovirus at 10 MOI (Ad-GFP and Ad-KLF4, respectively; purchased
from Research-Bio, Shanghai, China). For loss-of-function study, HUVECs were transfected with KLF4-specific siRNA (the targeting sequence is: CCA GAG GAG CCC AAG CCA ATT) or scrambled control oligonucleotides (50nM; purchased from GenePharma, Shanghai, China) using Lipofectamine 3000 according to the manufacturer’s instructions.

**Luciferase reporter gene assays**

The NF-κB reporter gene assay was conducted by co-transfection of Ig-κB-luciferase reporter gene plasmids (pTK81-IgK, 200 ng per transfection) with a *Renilla* luciferase vector (pRL, 20 ng per transfection) as described previously. For assessing the activity of miR-1 on KLF4 gene expression, a commercially available vector of pMIR-REPORT (Promega, Madison, USA) was re-constructed with a fragment of the 3’-UTR of KLF4 mRNA containing the putative miR-1 binding sequence as reported previously. Luciferase activity assays were performed using a dual-luciferase reporter assay system (Promega) and normalized relative to *Renilla* luciferase activity as described previously. Each experiment was performed in triplicate and repeated independently for at least three times.

**Animal Experiments**

Animal studies were approved by the Animal Use and Care Committee of Fudan University and were in conformity with the US Public Health Service Policy on Humane Care and Use of Laboratory Animals. All mice were housed in a temperature-controlled pathogen-free environment on a 12-hour light/dark cycle, had ad libitum access to food and water. For isolation of primary hepatocytes, C57/BL6J mice (male, aged 6-8 weeks) were randomly assigned to 2 groups (6/group), fed ad libitum with regular chow and a high-fat diet (HFD, containing 60 kcal% fat, 20% protein and 20% carbohydrate; Research Diets, #D12492, New Jersey), respectively, for 12 weeks. The details of diet compositions are shown in Supplementary Table 1.
ApoE−/− mice (male, aged 6-8 weeks) were purchased from Vital River Laboratory Animal Technology (Beijing, China) and fed with HFD for 10 weeks. Four weeks after HFD feeding, mice were injected intraperitoneally with AntagomiRs (Ribobio, Guangzhou, China) at a dose of 20 mg/kg wt in 0.2 ml saline once every week for 6 weeks. A scramble antagomiR (anta-Ctl) was used as control. Sequences of miR-1 antagomiR (antaR-1) are 5’-AUACAUACUUCUUUACAUUCCA-3’ and anta-Ctl 5’-CAGUACUUUGUGUAGUACAAA-3’.

Atherosclerosis Analysis

The experimental mice were perfused by cardiac puncture with 4% (w/v) paraformaldehyde to wash out blood from heart and all vessels after euthanasia. After removing the surrounding fat and connective tissues, the entire aorta was examined under stereomicroscope. For en face analysis, the whole aorta was excised from the aortic arch to the common iliac artery and stained with Oil red O. To analyze the atherosclerotic lesions, cross-sections of the aortic root were stained with hematoxylin-eosin (HE), Oil red O and Masson’s reagent, respectively. For immunohistochemistry assays, the frozen slides were blocked with 5% goat serum albumin and probed with the primary antibodies overnight at 4°C. The reactivity of the antibodies in the slides was detected using a streptavidin-peroxidase histostain-SP kit (ZsBio, Beijing, China). The peroxidase activity was visualized with diaminobenzidine, followed by haematoxylin staining to counterstain the nuclei. Images of each section were obtained with a bright-field microscope (Leica, DM6000B; Leica Microsystems) connected to a Leica CCD camera (DFC365FX), and quantified using IMAGEPRO PLUS software.

Statistical Analysis
All data were analyzed with SPSS 19.0. Comparisons between two groups were conducted using 2-tailed Student's t test or Mann-Whitney test, and ANOVA with Tukey’s post-hoc tests were performed for statistical significance among multiple groups. The level of statistical significance was set at P<0.01.
RESULTS

Characterization of EVs derived from steatotic hepatocytes

In an attempt to identify EVs released from hepatocytes under steatotic conditions, we applied a common cellular model of hepatosteatosis, in which hepatocytes were chronically exposed to a saturated free fatty acid, palmitic acid (PA). In line with our previous report\(^1\), treatment of Huh7 hepatocytes with PA resulted in a significant accumulation of lipids in a dose-dependent manner (Supplementary Fig 1A-1B). Because PA can also cause apoptosis in hepatocytes under certain conditions\(^2\), we sought to establish treatment conditions prior to EV collection that minimized the presence of apoptotic bodies. We found that exposing Huh7 hepatocytes to 0.2mM PA for 18 hrs stimulated significant intracellular lipid accumulation but did not induce evident cell death (Supplementary Fig 1C). Following this treatment, we collected EVs from cell-culture media by differential ultracentrifugation and characterized them by nanoparticle tracking analysis (NTA). In agreement with previous reports\(^3\), PA treatment resulted in nearly a 10-fold increase in EV released from Huh7 hepatocytes, compared to cells treated with vehicle alone (Fig 1A-1B). NTA revealed that the size distribution of EVs was 30–200 nm in diameter (Fig 1A). PA treatment slightly increased the mean size of the EVs in comparison with the controls (131nm vs 121nm, Fig 1A, 1C). The characteristic morphologies of EVs were further determined by transmission electron microscopy examination as shown in Fig 1D. The EVs were enriched in the characteristic markers of exosome, including Tsg101, CD63 and CD81, without expression of the proteins associated with other cellular organelles, such as early endosomes (EEA-1) and endoplasmic reticulum (Grp78) (Fig 1E), suggesting that the majority of EVs that we collected were likely exosomes derived from hepatocytes.

Steatotic hepatocyte-derived EVs induce endothelial cell inflammatory injury
To investigate the role of EVs in interaction of hepatocytes with vascular endothelial cells, we firstly examined whether hepatocyte-derived EVs can be taken in by endothelial cells. We applied a fluorescent dye, PKH26, to label the EVs derived from PA-treated (PA$^{EV}$) or control (Ctl$^{EV}$) Huh7 hepatocytes. As shown in Fig 2A, the red fluorescence clearly appeared within HUVECs following treatment with PKH26-labeled EVs for 12hrs, and the intracellular fluorescence was further increased at 24 hrs after treatment, revealing that endothelial cells can efficiently assimilate hepatocyte-derived EVs. Moreover, the density of intracellular fluorescence was significantly higher in the PA$^{EV}$-treated HUVECs than Ctl$^{EV}$-treated cells (Fig 2A), suggesting that EVs derived from steatotic hepatocytes could be more preferentially delivered to endothelial cells.

Having shown the ability of endothelial cells to take in hepatocyte-derived EVs, we then assessed the effect of EVs on the recipient endothelial cells. To this end, HUVECs were treated for 24 hr with PA$^{EV}$ or Ctl$^{EV}$ and analyzed using a Human Endothelial Cell Biology Profiler PCR array, which contains 84 key genes relevant to angiogenesis, permeability, inflammation and other endothelial functions. By comparison between the PA$^{EV}$-treated and Ctl$^{EV}$-treated groups, we found that of the 84 genes, 33 were differentially expressed (Supplementary Table 2). Among them, the top 10 up-regulated genes (≥2-fold) by PA$^{EV}$ were mostly related to endothelial inflammation, including E-selectin, ICAM-1, VCAM-1, IL-1β and MMP-1 (Fig 2B). The PA$^{EV}$ treatment-induced up-regulation of these pro-inflammatory molecules was further validated by qPCR analysis as shown in Fig 2C. Because NF-κB is regarded as a master controller of inflammatory gene expression, we then examined the effect of hepatocyte-derived EVs on the NF-κB pathway. Remarkably, treatment of HUVECs with PA$^{EV}$ resulted in significant increases in p65 phosphorylation and IκB degradation, compared to the Ctl$^{EV}$-treated cells (Fig 2D), indicating NF-κB pathway activation. To verify the effect of hepatocyte-derived EVs on NF-κB activation, we conducted a NF-κB-specific reporter gene assay. Serving as a positive control, TNFα
treatment resulted in a >20-fold increase in reporter activity (Fig 2E), which is consistent with our previous report. Notably, treatment with PA_EV resulted in a significant increase in reporter gene activity under both control- and TNFα-stimulated conditions in comparison with Ctl_EV (Fig 2E), further indicating the NF-κB pathway activation by the steatotic hepatocyte-derived EVs.

To further elucidate the effect of steatotic hepatocyte-derived EVs on endothelial cells, we isolated primary hepatocytes from a well-established animal model of NAFLD, i.e., HFD-induced obese (DIO) mice. Consistent with our previous report, hepatocytes isolated from DIO mice exhibited evident accumulation of lipid droplets and a significant increase in intracellular triglycerides, in comparison with cells isolated from control mice fed with a normal chow diet (Supplementary Fig 2). We co-cultured these primary hepatocytes with murine aortic endothelial cells. In keeping with the above findings in PA-treated Huh7 cells, co-culture with the fatty liver-derived hepatocytes resulted in a significant increase in E-selectin, ICAM-1 and VCAM-1 expression (Fig 2F) and NF-κB activation (Fig 2G) in endothelial cells, compared with cells co-cultured with normal hepatocytes. Remarkably, pre-treating hepatocytes with GW4869, a neutral sphingomyelinase inhibitor that effectively blocks EV release, almost completely abolished the effect of co-culture with fatty liver-derived hepatocytes on endothelial cells (Fig 2F-G). Collectively, the data suggest steatotic hepatocytes potently promote endothelial inflammation through the release of EVs.

The expression profile of EV miRNA is altered by steatotic hepatocytes with an up-regulation of miR-1

To identify the components responsible for the pro-inflammatory effect of EVs, we assessed the expression profiles of miRNA in the EVs derived from primary hepatocytes isolated from DIO (DIO_EV) and normal control mice (NC_EV) by Illumina
miRNA deep-sequencing. A wide range of changes in the EV miRNA expression was observed between NC\textsuperscript{EV} and DIO\textsuperscript{EV} groups. Among the differentially expressed miRNAs, the top 20 miRNAs with a pronounced significant difference (fold change > 2) between NC\textsuperscript{EV} and DIO\textsuperscript{EV} are illustrated in a heat map shown in Fig 3A (detailed in Supplementary Table 3). Of note, the cluster of miR-1, including miR-1a-3p and miR-1b-5p, was markedly up-regulated with the most significance in DIO\textsuperscript{EV}, compared to NC\textsuperscript{EV}. The increased miR-1 expression in steatotic hepatocyte-derived EVs was confirmed by qPCR analysis (Fig 3B). Interestingly, elevated miR-1 expression was also observed in the total liver tissues and primary hepatocytes isolated from DIO mice, compared to that of control mice (Fig 3C). However, the extent of miR-1 up-regulation in DIO\textsuperscript{EV} was higher than that in the liver tissues and hepatocytes (Fig 3B-3C), suggesting a selective enrichment of miR-1 in the EVs derived from steatotic hepatocytes. According to the database of “miRBase”, murine miR-1 corresponds to miR-1-3p, miR-206 and miR-1-5p in humans. Similar to the expression profile of murine miR-1, all the three members of human miR-1 were significantly elevated in the EVs derived from PA-treated human Huh7 hepatocytes (PA\textsuperscript{EV}), compared with that from vehicle-treated cells (Ctl\textsuperscript{EV}) (Fig 3D). Together, these data demonstrate that the miRNA expression profile in EVs was altered by steatotic hepatocytes with a significant up-regulation of miR-1 expression.

**Micro RNA miR-1 Promotes Endothelial Inflammation**

Given the pro-inflammatory effect of steatotic hepatocyte-derived EVs and significant up-regulation of miR-1 in EVs, we sought to test whether miR-1 mimics EV-induced endothelial inflammation. To this end, HUVECs were transfected with miR-1 or miR-206 using a lentiviral transfection method. A >90% transfection efficacy was achieved and a significant up-regulation of miR-1 or miR-206 detected following the transfection (Supplementary Fig 3). Remarkably, overexpression of miR-1 or miR-206 resulted in a significant increase in both mRNA and protein levels.
of E-selectin, ICAM-1 and VCAM-1 in HUVECs following TNFα stimulation (Fig 4A-4D). Correspondingly, TNFα-induced NF-κB activation and adhesion of monocytes on endothelial cells was also significantly increased by overexpression of miR-1 or miR-206 (Fig 4C-4E), indicating a potent pro-inflammatory effect of miR-1. We did not find any miR-1 binding sites for the three adhesion molecules within the mRNA sequences by miRNA target prediction analysis. However, among the predicted targets, the zinc finger protein Krüppel-like factor 4 (KLF4) was brought to our attention. A miR-1 binding site within the 3’ untranslated region (3’ UTR) of KLF4 was identified based on bioinformatics analysis (Fig 4F), which is consistent with a previous report 23. In fact, miR-1 has been shown to directly bind to and suppress KLF4 expression in smooth muscle cells 23. In line with this, KLF4 expression was significantly down-regulated by overexpression of either miR-1 or miR-206 in HUVECs (Fig 4G). Moreover, enforced overexpression of KLF4 dramatically abolished the miR-1-induced VCAM-1 expression, whereas knockdown of KLF4 expression by its specific siRNA was able to mimic and potentiate the effect of miR-1 (Fig 4H). Collectively, these data suggest that KLF4 is likely a functional target of miR-1.

To determine whether KLF4 is indeed a direct target of miR-1, we conducted KLF4 reporter gene assays. Strongly substantiating a direct effect of miR-1 on KLF4, co-transfection of miR-1-mimics with the KLF4 3’-UTR reporter resulted in a significant reduction of the luciferase activity in a dose-dependent manner (Fig 4I).

Hepatocyte-Derived EVs Promote Endothelial Inflammation via miR-1

Having demonstrated the pro-inflammatory effect of miR-1, we hypothesized that EV-derived miR-1 mediates hepatocyte-induced endothelial inflammation under the steatotic conditions. To verify this hypothesis, we first examined whether miRNAs contained in hepatocyte-derived EVs are effectively delivered to endothelial cells. To this end, a fluorescent Cy3-labeled miR-1 mimic was overexpressed in Huh7 cells that were then co-cultured with HUVECs. After 24-hr co-culture, the fluorescent dye
was evidently accumulated within HUVECs (Fig 5A). Quantitative analysis of intracellular miR-1 expression showed that while the endogenous miR-1 expression was barely detected in endothelial cells, it was dramatically elevated in HUVECs co-cultured with either control or miR-1 transfected Huh7 hepatocytes (Fig 5B). Remarkably, by blocking EV release from the hepatocytes, GW4869 virtually abolished the increased miR-1 in HUVECs (Fig 5B), indicating hepatocytes can deliver miR-1 into endothelial cells through the release of EVs. In addition, exposure of HUVECs to PA$^{EV}$ resulted in a similar increased level of miR-1 expression, compared to the control EV treatment (Fig 5C). Of note, treatment of HUVECs with the anti-miR-1 inhibitor significantly attenuated PA$^{EV}$-induced NF-κB activation (Fig 5D). Furthermore, the PA$^{EV}$-induced suppression of KLF4 was reversed by the inhibition of miR-1 (Fig 5D). Taken together, these data illustrate that miR-1 is instrumental in mediating the pro-inflammatory effect of steatotic hepatocyte-derived EVs on endothelial cells.

**Inhibition of miR-1 attenuated atherogenesis in ApoE$^{-/-}$ mice**

Because endothelial inflammation plays a critical role in the development of atherosclerosis, we aimed to investigate the pro-inflammatory effect of miR-1 *in vivo*. To this end, we employed ApoE$^{-/-}$ mice fed with HFD, a well-established animal model of atherosclerosis accompanied with fatty liver, and treated the mice with antagomiR-1 that specifically blocks miR-1 function. Consistent with the above observations *in vitro*, the inflammatory phenotypes, including suppressed expression of KLF4, activation of NF-κB and up-regulation of VCAM-1, were significantly attenuated in mouse aortic tissues following antagomiR-1 treatment (Fig 6A), which confirmed the efficacy of antagomiR-1 in the experimental animals. There were no significant changes in general metabolic characteristics, including body weight, adiposity and levels of blood glucose, triglycerides and cholesterol, between antagomiR-1-treated group and the control group that was administered a scrambled
antagomiR (see Supplementary Fig 4). Notably, both the number and size of atherosclerotic plaques in the aortic arch, thoracic aorta region and whole aorta were significantly decreased by antagomiR-1 treatment, compared with control group (Fig 6B). Histological analysis of cross-sections by HE staining and Oil red O staining on the proximal aorta revealed significantly less lesion areas in the antagomiR-1-treated mice than that in the controls (Fig 6C). Furthermore, plaque composition analysis was determined by quantification of smooth muscle actin (SMA), collagen content and ICAM-1 expression. As shown in Fig 6D, SMA levels were significantly decreased by antagomiR-1 treatment compared with control treatment, indicating an ability of antagomiR-1 to suppress vascular smooth muscle cell growth within the plaques and attenuate atherosclerotic lesions. In contrast, the collagen content was increased, suggesting that antagomiR-1 treatment led to more stable plaques (Fig 6E). We also investigated ICAM-1 expression as a marker of endothelial inflammation, and found that ICAM-1 was significantly decreased by antagomiR-1 treatment (Fig 6F). Taken together, these results reveal that inhibition of miR-1 profoundly ameliorated the formation and progression of atherosclerotic plaques in ApoE−/− mice.

Discussion

In the current study, we provide both experimental evidence and mechanistic data demonstrating that steatotic hepatocytes are capable of promoting endothelial inflammation through EV release. The findings provide an insight into an important role for EVs in the interaction between the liver and the vasculature, suggesting a mechanistic link between NAFLD and atherosclerotic CVD.

Following intensive investigations in recent years, EVs have emerged as an important mediator of intercellular and inter-organ communications, playing critical roles in a wide-range of pathophysiological processes.10,26 EVs can be released into extracellular fluid and the circulation by various types of cells, such as macrophages, hepatocytes, adipocytes and many others.9,11 The number and overall composition of
EVs released by donor cells are often altered in response to various stresses or pathological stimuli \(^{10, 26}\). For instance, hepatocytes under lipotoxic stress have been shown to dramatically increase the release of EVs \(^{24, 27-29}\). In line with previous reports \(^{24, 29}\), we found that the release of EVs from steatotic hepatocytes, induced by PA treatment or isolated from DIO mice, is increased by about 10-fold compared to their respective control cells. Moreover, through high throughput sequencing of small RNAs, we found that over 500 miRNAs present within EVs are differentially expressed between normal and steatotic hepatocytes, suggesting a pathological role for EVs in hepatocytes. Indeed, it has been reported that hepatocyte-derived EVs can promote inflammation and fibrosis in the liver by activating local macrophages in a paracrine manner, impelling the progress of NAFLD towards NASH \(^{24, 27-29}\). We have demonstrated herein that the EVs released by hepatocytes act as a signal, mediating distant communication between liver and vascular endothelium under NAFLD conditions. This notion is supported by the following findings. Firstly, treatment of HUVEC with hepatocyte-derived EVs labeled with PKH26 resulted in marked accumulation of red fluorescence within cells in a time-dependent fashion (Fig 2A), indicating endothelial cells can efficiently uptake hepatocyte-derived EVs. Moreover, the EVs derived from steatotic hepatocytes appear to be preferentially taken up by endothelial cells. Secondly, exposure of endothelial cells to steatotic hepatocyte-derived EVs led to a significant increase in the expression of pro-inflammatory molecules, including E-selectin, VCAM-1, ICAM-1, IL-1β and MMP-1, and activation of the NF-κB pathway (Fig 2B-2E). Thirdly, this pro-inflammatory effect of steatotic hepatocyte-derived EVs can be mimicked by co-culture of endothelial cells with steatotic hepatocytes (Fig 2F-2G). Finally, once EV release was blocked by GW4869 in the co-culture system, the pro-inflammatory effect was profoundly suppressed (Fig 2F-2G). Taken together, these findings indicate that the steatotic hepatocytes are primed to deliver pro-inflammatory signals into endothelial cells via EV release.
Among the various active components within EVs, miRNAs have gained particular attention. In fact, EVs have recently emerged as a principal carrier for extracellular miRNAs that signal in intercellular communications. Upon profiling hepatocyte-derived EV miRNAs, we found that a set of miRNAs was significantly altered by steatotic hepatocytes, in comparison with control cells. Specifically, a significant increase in miR-1 expression was identified in the steatotic hepatocyte-derived EVs. Interestingly, although miR-1 expression was also increased in the total liver tissues and primary hepatocytes derived from DIO mice, the extent of miR-1 up-regulation in EVs was more evident (Fig 3B-3C), suggesting a selective increase of miR-1 expression by the EVs derived from steatotic hepatocytes.

Originally described as a muscle-specific miRNA, miR-1 is highly expressed in muscle cells, including smooth muscle, skeletal muscle and cardiac muscle, and plays a critical regulatory role in muscle cell differentiation and growth. An elevated circulating level of miR-1 was found to associate with myocardial steatosis and suggested to be a hallmark of diabetic cardiomyopathy. In addition, miR-1 has been reported to contribute to glucose-mediated apoptosis in cardiomyocytes by down-regulating LXRα expression. Furthermore, miR-1 expression has been recently found in the vascular endothelium within malignant tumors, suggesting a potential role of miR-1 in tumor angiogenesis. However, the physiologic function of miR-1 in endothelial cells, especially that relevant to cardiometabolic disease, has not yet been explored. In the present study, we found that hepatocyte-derived EVs contained a relatively high expression level of miR-1 family mRNAs, including miR-1-3p, miR-1-5p and miR-206. Of note, miR-1 expression in EVs derived from steatotic hepatocytes was significantly higher than that from control hepatocytes (Fig 3A-3D). Interestingly, while the endogenous level of miR-1 expression was nearly undetectable in endothelial cells under basal normal conditions (Fig 5B), it was dramatically increased in the cells either treated with steatotic hepatocyte-derived EVs (Fig 5C) or co-cultured with hepatocytes (Fig 5B). By blocking EV release from the
hepatocytes, GW4869 almost completely abolished endothelial expression of miR-1 (Fig 5B), corroborating the ability of endothelial cells to uptake the miR-1 extracted from steatotic hepatocyte-derived EVs. Remarkably, overexpression of miR-1 in endothelial cells mimicked the pro-inflammatory effect of EVs, as reflected by a significant increase in TNFα-stimulated E-selectin, VCAM-1 and ICAM-1 expression, monocytes adhesion and NF-κB activation (Fig 4A-4E). By contrast, the pro-inflammatory effect was profoundly blocked by the miR-1-specific inhibitor (Fig 5D). Collectively, these data uncover an important role of miR-1 in mediating the pro-inflammatory effect of steatotic hepatocyte-derived EVs in vascular endothelial cells.

To further investigate the role of miR-1 in endothelial inflammation and its significance in cardiometabolic disease, we employed a well-established animal model of atherosclerosis accompanied by fatty liver, i.e., ApoE−/− mice fed with HFD. As expected, numerous atherosclerotic plaques were formed in the aortic arch, thoracic aorta region and whole aorta in ApoE−/− mice after 10-weeks feeding with HFD (Fig 6B). Remarkably, treatment with antagomiR-1 that specifically blocks miR-1 function (Fig 6A) resulted in a significant reduction in both the number and size of the atherosclerotic plaques throughout the whole aorta, compared with the control treatment (Fig 6B). Furthermore, histological analysis of cross-sections of the proximal aorta revealed that the antagomiR-1-treated mice developed significantly less atherosclerotic lesion areas than the control group (Fig 6B-6C), along with a reduced level of smooth muscle actin (Fig 6D), increased content of collagen fibers (Fig 6E), and less VCAM-1 positive plaques (Fig 6F). These data illustrate that the antagomiR-1-induced inhibition of miR-1 suppressed vascular smooth muscle cell growth, stabilized plaques and reduced endothelial inflammation, leading to marked amelioration of atherosclerotic plaque formation and progression in ApoE−/− mice. The findings thus delineate an important role of miR-1 in the development of atherosclerosis in ApoE−/− mice. Given the fact that steatotic hepatocyte-derived EVs
induced endothelial inflammation *in vitro* via their cargo of miR-1, we speculate a similar mechanism may promote atherogenesis in NAFLD. However, due to the experimental limitations, this rational has not been directly clarified in this study. It is also worth noting that although the anti-atherogenic effect of antagomiR-1 appears chiefly attributable to the inhibitory effect on endothelial inflammation, we cannot exclude other possible actions independent of endothelial cells.

The major biological function of miRNAs is post-transcriptional regulation of gene expression, and they are thus involved in many fundamental cellular processes. KLF4 belongs to a subfamily of the zinc finger class of transcriptional regulators, which has been demonstrated as an important regulator of vascular homeostasis. By using endothelial-specific gain-of-function and loss-of-function approaches *in vivo*, a recent study has identified a key protective role of KLF4 against endothelial inflammation and atherothrombosis. According to bioinformatic analysis, a miR-1 binding site exists in the 3’UTR of KLF4. Indeed, miR-1 has been shown to directly bind to and suppress KLF4 expression in smooth muscle cells. In keeping with this finding, we found that overexpression of miR-1 significantly down-regulated KLF4 expression in endothelial cells. Moreover, enforced overexpression of KLF4 profoundly blocked the pro-inflammatory effect of miR-1, whereas knockdown of KLF4 expression potentiated the effect, indicating that KLF4 is likely a functional target of miR-1. Likewise, the endothelial cells treated with steatotic hepatocyte-derived EVs resulted in a similar suppression of KLF4. Moreover, the EV-reduced KLF4 expression was significantly abolished by the anti-miR-1 inhibitor, suggesting that the pro-inflammatory effect of miR-1 is mediated through KLF4 suppression. The KLF4 reporter gene assays reveal that co-transfection of miR-1-mimics with the KLF4 3’-UTR reporter resulted in a dose-dependent suppression of luciferase activity, further indicating that KLF4 is a direct target of miR-1. KLF4 is known to interact with p300, a key coactivator required for optimal transcriptional activity of many factors, including...
NF-κB and its target genes such as VCAM-1 and tissue factor\textsuperscript{35,37}. In accordance with this, we found that both steatotic hepatocyte-derived EVs and miR-1 treatment resulted in significantly reduced KLF4 expression, accompanying NF-κB activation and elevated adhesion molecule expression in endothelial cells. Therefore, it is interesting to postulate a signaling model, i.e., the miR-1-KLF4-NF-κB pathway, by which steatotic hepatocyte-derived EVs promote endothelial inflammation and atherogenesis.

In conclusion, our study provides compelling evidence implicating hepatocyte-derived EVs as key mediators of distant communications between the liver and vasculature, especially in the context of NAFLD, shedding light on a mechanism underlying the development of cardiometabolic disease.
Acknowledgments

This work was supported by grants from National Natural Science Foundation of China (81561128014 and 81870559 to P.X.) and Fudan Distinguished Professorship (to P.X.). We thank Dr. Carol Wadham (South Australia Pathology, Adelaide, Australia) for proofreading of the manuscript.
References


Figure legends

Figure 1. Characterization of hepatocyte-derived EVs. Huh7 cells were treated for 18 hrs with 0.2mM PA or vehicle alone (Ctl), and then EVs were isolated by differential ultracentrifugation from the conditioned medium and normalized to cell numbers (referred to as PA$^{EV}$ and Ctl$^{EV}$, respectively). (A) Concentration and size (nm, nanometers) distribution of EVs were determined by NTA, and quantified as in (B) and (C), respectively. Data are expressed as means ± SD (n=3). *P<0.01, **P<0.001 (Student’s t test). (D) Representative electron microscopic photographs of Ctl$^{EV}$ and PA$^{EV}$, scale bar =100 nm. (E) Representative Western blots showing the expression of exosome markers, including CD63, CD81 and Tsg101 and the negative control GRP78 and EEA1 in total cell lysate and EVs.

Figure 2. Effects of hepatocyte-derived EVs on endothelial cells. Hepatocyte-derived EVs were isolated from the same number of Huh7 cells treated for 18 hrs with 0.2mM PA (PA$^{EV}$) or vehicle (Ctl$^{EV}$) as described in Fig 1. (A) Ctl$^{EV}$ and PA$^{EV}$ were labeled with PKH26 and then incubated with HUVECs. After incubation for 12 hrs, HUVECs were washed, fixed and counterstained with DAPI. Representative fluorescent micrographs show hepatocyte-derived exosomes internalized by HUVECs, and the quantitative data are shown in the left bar chart. (B) HUVECs were treated for 24 hrs with Ctl$^{EV}$, PA$^{EV}$ or without treatment (Blank), and then the extracted total RNA was subjected to analysis by Human Endothelial Cell Biology RT2 Profiler™ PCR array. The heat map shows the top 10 up-regulated genes in PA$^{EV}$-treated HUVECs, compared to Ctl$^{EV}$-treated cells. (C) Levels of E-selectin, VCAM-1, ICAM-1, IL1β and MMP1 mRNA were analyzed by qPCR in HUVECs treated with Ctl$^{EV}$, PA$^{EV}$ or without treatment (Blank). (D) Levels of phosphorylated-p65 (p-p65) and IκB were determined by Western blotting in Ctl$^{EV}$ and PA$^{EV}$-treated HUVECs. The bottom chart shows quantifications from the blots.
normalized to the intensity of the loading control β-actin. (E) NF-κB-dependent gene transactivation was measured in HEK293 cells cotransfected with Ig-κB-luc reporter plasmid and Renilla luciferase control vector. The transfected cells were treated for 24 hrs with CtlEV, PAEV or without treatment (Blank) followed by TNF (1 ng/ml) treatment for 4 h, and then the reporter gene activity was determined and normalized relative to Renilla luciferase activity (RLA). (F) Relative mRNA levels of E-sele, ICAM-1 and VCAM-1, and (G) levels of p-p65 and IκB were determined in murine aortic endothelial cells that were co-cultured for 18 hrs with primary hepatocytes isolated from normal control (NC) or DIO mice. The bottom chart shows quantifications from the blots normalized to the intensity of β-actin. GW indicates the presence of GW4869 in co-culture system. Data are expressed as means ± SEM (n=3-4). *P<0.01, **P<0.001 (A-E: Student’s t test; F-G: ANOVA test).

Figure 3. Expression profiles of miRNA in hepatocyte-derived EVs. (A) The heat map shows differential expression of miRNAs between hepatocyte-derived EVs isolated from normal control (NC\textsuperscript{EV}) and DIO (DIO\textsuperscript{EV}) mice. (B) Levels of mmu-miR-1a-3p in NC\textsuperscript{EV} and DIO\textsuperscript{EV} and (C) in the liver tissues and murine primary hepatocytes (MPH) isolated from the experimental animals were analyzed by qPCR. (D) Levels of hsa-miR-206, hsa-miR-1-3p and hsa-miR-1-5p in were determined by qPCR in EVs derived from PA (PA\textsuperscript{EV}) or vehicle-treated (Ctl\textsuperscript{EV}) Huh7 cells. Data are expressed as means ± SEM (n=4-6). *P<0.01, **P<0.001 (Mann-Whitney test).

Figure 4. Pro-inflammatory effect of miR-1 on endothelial cells. HUVECs were transfected with miR-1 (A,C) or miR-206 (B,D) using a lentiviral transfection method. Following treatment with or without TNFα (1ng/ml) for 6 hrs, (A,B) levels of E-selectin, ICAM-1 and VCAM-1 mRNA were measured by qPCR, and (C,D) Western blotting for IκB, ICAM-1 and VCAM-1, and (E) adhesion of monocytes to
the treated HUVECs was analyzed. The quantitative data for the blots normalized to the intensity of β-actin and for monocyte adhesion of were shown in the bottom and right charts, respectively. (F) The predicted binding sites of miR-1 in the 3’ UTR of human KLF4 gene. (G) Levels of KLF4 expression in HUVECs overexpressing miR-1 or miR-206 were analyzed by Western blotting, and the blot quantitation shown in the bottom chart. (H) HUVECs were infected with control (Ad-GFP) or KLF4 expression adenovirus (Ad-KLF4) at 10 MOI, or transfected with siRNA specifically targeting KLF4 (siR-KLF4) or non-specific control siRNA (siR-NC) at 50nM. After TNFa (1ng/ml) treatment for 6 hrs, levels of KLF4, IkB and VCAM-1 were measured by Western blotting. The bottom chart shows quantifications from the blots normalized to the intensity of β-actin. (I) KLF4 reporter gene activity was measured in HEK293 cells cotransfected with Renilla luciferase and pMIR-REPORT vector constructed with a fragment of the 3’-UTR of KLF4 mRNA containing the putative miR-1 binding sequence. After 18 hrs transfection, the cells were re-transfected with miR-1 mimics at 25, 50 and 100 nM or control miRNA (NC), and then the reporter gene activity was determined at 18 hrs after miRNA transfection and normalized relative to Renilla luciferase activity (RLA). Data are expressed as means ± SEM (n=3-4). *P<0.01, **P<0.001 (A-G and I: Student’s t test; H: Mann-Whitney test).

Figure 5. Effect of hepatocyte-derived EV miR-1 on endothelial cells. (A) Huh7 cells were transfected with Cy3-labeled miR-1 mimics and then co-cultured with HUVECs. After co-culture for 24 hrs, HUVECs were washed, fixed and counterstained with DAPI. Representative micrographs show hepatocyte-derived Cy3-labeled miR-1 (red fluorescence) that was internalized within HUVECs. (B) HUVECs were co-cultured for 24 hrs with control (Huh7Ctl) or miR-1-transfected Huh7 hepatocytes (Huh7mir1) or without co-culture (Nil) in the presence or absence of GW4869, and then levels of miR-1 were determined by qPCR. (C) Levels of miR-1
and miR-206 were determined by qPCR in HUVECs treated for 18 hrs with EVs derived from PA (PA\textsuperscript{EV}) or vehicle-treated (Ctl\textsuperscript{EV}) Huh7 cells. (D) Levels of p-p65, IκB and KLF4 were analyzed by Western blotting in Ctl\textsuperscript{EV} and PA\textsuperscript{EV}-treated HUVECs in the presence or absence of anti-miR-1 inhibitor transfection (antiR1). The bottom chart shows quantifications from the blots normalized to the intensity of β-actin. Data are expressed as means ± SEM (n=4-6). *P<0.01, **P<0.001 (C: Student’s t test; B and D: ANOVA test).

Figure 6. Effect of miR-1 inhibition on atherosclerosis in ApoE\textsuperscript{-/-} mice. ApoE\textsuperscript{-/-} mice fed with HFD were treated with miR-1 antagonir (antaR-1) or a scramble antagonir (antaCtl), respectively (n = 6 mice per group). (A) Expression levels of KLF4, IκB and VCAM-1 in aorta of the antagonir-treated mice were analyzed by Western blotting, and the quantifications of the blots normalized to β-actin shown in the bottom chart. (B) The images show Oil-red O staining of the entire aorta and atherosclerotic lesion area analyzed by image J (shown in the right chart). (C) Representative microscopic images of HE and Oil-red O staining at the aorta root. (D) Collagen content in atherosclerotic plaques was assessed with Masson’s staining. Representative micrographs display the immunohistological staining for SMA (E) and VCAM-1 (F) in cross-sections of the proximal aorta. Bar charts show the positive staining area analyzed by image J. Original magnification, x20 (C); x100 (D-F). Data are expressed as mean ± SD (n=6; one was omitted in (F)). *P<0.01, **P<0.001 (Mann-Whitney test).

Journal of Hepatology
CTAT methods
Tables for a “Complete, Transparent, Accurate and Timely account” (CTAT) are now mandatory for all revised submissions. The aim is to enhance the reproducibility of methods.
Graphical abstract

NAFLD

Hepatocyte-derived EVs

miR-1

KLF4↓ NF-κB↑

Endothelial inflammation

Atherogenesis

Distant Communication
Figure 1

A. Concentration vs. Size (nm) for CtlEV and PAEV

B. EV released (fold change) for CtlEV and PAEV

C. EV mean size (nm) for CtlEV and PAEV

D. CtlEV and PAEV Cell Lysate images

E. Western blot analysis of CD63, Tsg101, CD81, EEA-1, and GRP78 in Cell Lysate and EV
Figure 2

A.  

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![Fluorescence Density (%)](image5)

**Fluorescence Density (%)**

- **CtlEV**
- **PAEV**

**12h**

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**24h**

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**Comparison**

- **CtlEV** vs **PAEV**

**Significance**

- **24h**
  - **CtlEV** vs **PAEV**: significant difference (p < 0.01)

**Legend**

- **CtlEV**: Control EV
- **PAEV**: Paired EV

**Graph**

- **Fluorescence Density (%)**
- **12h**
- **24h**
- **CtlEV**
- **PAEV**
- **Significance** (p < 0.01)
Figure 2

B. Relative mRNA levels for SELE, VCAM-1, ICAM-1, CDH5, SPHK1, PROCR, SERPINE1, HOMX1, IL-1B, and MMP-1 across Blank, Ctl, EV, PAlv, ChEv, and PAEv conditions.

C. Graphic representation of relative mRNA levels for E-sel, VCAM, ICAM, IL1, and MMP1, showing significant differences between conditions.
Figure 2

D. 

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E. 

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<td>TNF</td>
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Arbitrary unit

0 1 2 3 4 5 6 7 8

0 20 40 60 80

Nil TNF
Figure 2

**F.** Relative mRNA levels of E-selectin (E-sel), VCAM, and ICAM for NC, NC+GW, DIO, and DIO+GW groups.

**G.** Western blots showing expression levels of p-p65, IκB, and actin for NC, NC+GW, DIO, and DIO+GW groups.
Figure 3

A. NC\textsuperscript{EV} DIO\textsuperscript{EV}

1 2 1 2

miR1a-3p
miR1b-5p
miR-140-3p
miR-126a-5p
miR-3470b
miR-181a-5p
miR126b-3p
miR-3535
miR-133b-3p
miR-344d-3p
miR-133a-3p
miR-143-3p
miR-149-5p
miR-1198-5o
miR-127-3p
miR-674-3p
miR-676-3p
miR-652-3p
miR-425-5p
miR-664-5p

B.

[Graph showing relative miRNA levels for NC and DIO groups.]

C.

[Graph showing relative miRNA levels for NC, DIO, PA\textsuperscript{EV}, and Ctl\textsuperscript{EV} groups.]

D.

[Graph showing relative miRNA levels for miR-1-3p, miR-206, and miR-1-5p groups.]
Figure 4

A. Relative mRNA level of E-sel, ICAM, and VCAM in Ctl, Ctl+TNF, miR-1, and miR-1+TNF conditions.

B. Relative mRNA level of ICAM, VCAM, and actin in Ctl, Ctl+TNF, miR-206, and miR-206+TNF conditions.

C. Western blot analysis of IκB, ICAM, VCAM, and actin in Ctl and miR-1 conditions with TNFα.

D. Western blot analysis of IκB, ICAM, VCAM, and actin in Ctl and miR-206 conditions with TNFα.
Figure 4

E.  
Ctl  
miR-1  

Ctl  
miR-206  

F.  
KLF4 3'UTR  5’GGAUGGAUCUUCUAU…CAUuccAA  
Has-miR-1-3p  3’UAUGUAUAGAAAUGUAGGU  
Has-miR-206  3’GGUGUGUGAAGGAUGUAGGU  

G.  

KLF4  
actin  

**
Figure 4

H. | Ad-GFP | Ad-KLF4 | siR-NC | siR-KLF4 |
--- | --- | --- | --- | --- |
| Ctl | miR-1 | Ctl | miR-1 |

- KLF4
- IκB
- VCAM
- actin

<table>
<thead>
<tr>
<th>KLF4</th>
<th>IκB</th>
<th>VCAM-1</th>
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</table>

**Arbitrary unit**

I. **Reporter activity (RLA)**

- NC
- miR-1

* * *
Figure 5

A. [Images of DAPI, Cy3, and merged images showing cell nuclei and Cy3 staining.]

B. [Bar chart showing relative miRNA levels with error bars for Nil, Huh7\textsuperscript{Ctl}, and Huh7\textsuperscript{miR1} with and without GW treatment.]

C. [Bar chart showing relative miRNA levels for miR-1 and miR-206 with error bars for Ctl\textsuperscript{EV} and PA\textsuperscript{EV}.]

D. [Western blots for p-p65, IκB, KLF4, and actin with protein levels for Ctl\textsuperscript{EV}, PA\textsuperscript{EV}, and PA\textsuperscript{EV}+antiR1 with error bars.]
Figure 6

A.

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**Arbitrary unit**

- KLF4: 0, 1, 1.5, **3, 2**
- IkB: 0, 2, 1, **1.5**
- VCAM: 0.5, 1, **1.5**

B.

Lesion area (%)

- antaCtl: 1, 1.5, 2
- antaR-1: 0.5, 1, **1.5**

*** ***
Figure 6

C. antaCtl  antaR-1

Lesion area (%)

***
Figure 6

D. antaCtl antaR-1

E.  

F.  

VCAM-1 (%)

SMA (%)

Collagen (%)

* 0.00 0.05 0.10 0.15 0.20 0.25

0.0 0.1 0.2 0.3 0.4

* 0.00 0.05 0.10 0.15 0.20 0.25

0.0 0.1 0.2 0.3 0.4

*