

Steatotic hepatocyte-derived extracellular vesicle miR-192 modulates M2-associated marker expression by suppressing IL6ST

MIAO WU¹, NAN LI²

¹Department of Hematology, Guangdong Provincial People's Hospital, Guangdong Academy of Medical Sciences, Southern Medical University, Guangdong, China

²Department of Immunology, West China School of Basic Medical Sciences and Forensic Medicine, Sichuan University, Chengdu, China

Abstract

Introduction: Extracellular vesicles, which transport microRNAs, are emerging biomarkers for metabolic dysfunction-associated steatotic liver disease. Although extracellular vesicles modulate nearby hepatocytes, their influence on macrophages – central to disease progression – remains unclear. We aimed to identify functional microRNAs in extracellular vesicles and elucidate their role in macrophage activation and polarization.

Material and methods: Extracellular vesicles were isolated from steatotic AML12 hepatocytes using ultracentrifugation and characterized by transmission electron microscopy. Cellular uptake was confirmed using PKH67 labeling and confocal microscopy. RAW264.7 macrophages were treated with extracellular vesicles or microRNA mimics. Predicted microRNA targets were validated using a dual-luciferase assay and quantitative real-time polymerase chain reaction (PCR). Macrophage polarization markers (M1/M2) were analyzed using quantitative real-time PCR. Statistical significance was set at $p < 0.05$ (GraphPad Prism).

Results: Extracellular vesicles from steatotic AML12 hepatocytes showed enrichment of microRNAs (e.g., mmu-miR-122, -21, and -34a), with mmu-miR-192 showing the greatest increase (2.2-fold; $p < 0.01$). These extracellular vesicles were taken up by macrophages. Treatment with extracellular vesicle-derived miR-192 suppressed IL6ST expression ($p < 0.05$) and inhibited expression of M1 markers (e.g., iNOS, TNF- α , and IL-1 β), while enhancing the expression of M2 markers (e.g., CD206 and IL-10) ($p < 0.05$). Restoring IL6ST expression reversed these effects.

Conclusions: Our findings suggest that extracellular vesicles may mediate miR-192 transfer from steatotic hepatocytes to macrophages in metabolic dysfunction-associated steatotic liver disease. By targeting IL6ST and shifting macrophage marker expression toward an M2-associated profile, the miR-192/IL6ST axis may contribute to the regulation of steatotic liver inflammation and warrants further investigation as a potential therapeutic target.

Key words: fatty liver, extracellular vesicle, microRNA, macrophage, glycoprotein 130.

(Cent Eur J Immunol 2026; 51:)

Introduction

Metabolic dysfunction-associated steatotic liver disease (MASLD) is a common chronic liver condition that contributes substantially to liver-related morbidity and mortality worldwide [1]. The disease is pathologically characterized by excessive lipid accumulation in hepatocytes (hepatic steatosis) and can progress to metabolic dysfunction-associated steatohepatitis, leading to hepatic inflammation and cellular damage [2]. MASLD can progress to fibrosis and cirrhosis [3] and may ultimately

lead to hepatocellular carcinoma [4]. It exacerbates other conditions such as cardiovascular disease and type 2 diabetes mellitus [5]. The increasing prevalence of MASLD underscores the pressing clinical challenges it poses. Although obesity, insulin resistance, alterations in gut microbiota composition, and genetic susceptibility have been recognized as risk factors for MASLD [6], the molecular mechanisms underlying MASLD pathogenesis remain unclear.

M1 to M2 macrophage polarization is commonly observed in tumor-associated macrophages during cancer

Correspondence: Prof. Nan Li, Department of Immunology, West China School of Basic Medical Sciences and Forensic Medicine, Sichuan University, No. 17, Section 3, Renmin South Road, Chengdu 610041, China, phone: +86-28-18628025479, fax: +86-28-85403260, e-mail: nanli_immunology@126.com
Submitted: 12.03.2026, Accepted: 18.08.2026

Copyright © Polish Society of Experimental and Clinical Immunology. This is an Open Access article distributed under the terms of the Creative Commons Attribution-NonCommercial-ShareAlike 4.0 International (CC BY-NC-SA 4.0). License (<http://creativecommons.org/licenses/by-nc-sa/4.0/>)

development [7]. In the liver, Kupffer cells, which constitute 80-90% of tissue macrophages, orchestrate essential processes in maintaining health and mediating disease development [8]. Their polarization states and associated inflammatory signaling are critically involved in MASLD pathogenesis [9]. However, the mechanisms underlying hepatocyte–Kupffer cell communication require further clarification.

Dysregulated microRNA (miRNA) expression has been implicated in various liver diseases [10], including MASLD [11]. Disease-specific miRNA profiles suggest their utility as biomarkers and therapeutic targets [12]. For example, studies comparing patients with varying stages of MASLD (from simple steatosis to advanced fibrosis) and healthy individuals have reported altered serum levels of miRNAs such as miR-122, miR-192, miR-34a, and miR-21, with some profiles correlating with disease severity or histological features [11]. Despite advances in our understanding of intracellular miRNA functions, the roles of extracellular miRNAs, particularly those secreted by hepatocytes, remain poorly defined. A significant proportion of circulating miRNAs is encapsulated in extracellular vesicles (EVs) [13]. Experimental evidence indicates that EVs containing miRNAs can be delivered to recipient cells, where they exert regulatory functions [14], influencing processes such as stress responses, immune modulation, and tumor behavior [15].

In MASLD, hepatocyte-derived EVs modulate steatotic hepatocyte function via miRNA transfer [16]. However, the role of such miRNAs in hepatocyte–macrophage communication remains unexplored. In this study, we aimed to investigate the role of EV-mediated communication in the hepatic microenvironment of MASLD, with a focus on the function of miR-192 in macrophage polarization. We also aimed to identify the mechanistic target through which miR-192 exerts its effects.

Material and methods

Hepatocyte culture

AML12 murine hepatocyte line (American Type Culture Collection, Manassas, VA, USA) was cultured in Dulbecco's Modified Eagle's Medium with Nutrient Mixture F-12 (DMEM/F12) (Sigma-Aldrich, St. Louis, MO, USA) containing 10% fetal bovine serum (Gibco, Thermo Fisher Scientific, Waltham, MA, USA). At 70-80% confluence, the cells were serum-starved for 24 h to induce quiescence. The cells were exposed to control or high free fatty acid (HFFA) medium (1.0 mM oleate-palmitate, 2 : 1, complexed with 1% fatty acid-free bovine serum albumin [Sigma-Aldrich]) for 24 h [17]. RAW 264.7 macrophages (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM with 10% fetal calf serum under standard conditions.

Steatosis assessment

The AML12 cells seeded in 96-well black plates (4×10^4 cells/ml) were stained with Nile Red (1 mg/ml) (Sigma-Aldrich) 24 h after the treatment. Fluorescence was measured at 488/510 nm, and lipid deposition was visualized using fluorescence microscopy (Nikon, Tokyo, Japan).

EV isolation

EVs were isolated from AML12 culture supernatants via differential centrifugation [18] at $300 \times g$ for 10 min, $2,000 \times g$ for 20 min, $10,000 \times g$ for 1 h, and $100,000 \times g$ for 70 min. The pellets were resuspended in water for further analysis.

EV characterization and uptake analysis

For morphological characterization, the isolated EVs were deposited on carbon-coated copper grids, negatively stained with phosphotungstic acid (Sigma-Aldrich) and imaged using an FEI Tecnai G2F20S-Twin field-emission transmission electron microscope (Thermo Fisher Scientific, Waltham, MA, USA). To assess EV uptake by macrophages, hepatocyte-derived EVs were labeled with PKH67 dye (Sigma-Aldrich), purified, and cocultured with RAW264.7 cells. After 24 h, the cell nuclei were counterstained with Hoechst 33258 (Thermo Fisher Scientific, Waltham, MA, USA), and EV internalization was examined using confocal microscopy (LSM800; Carl Zeiss, Jena, Germany).

miRNA expression analysis

RNA was extracted from EVs or the recipient RAW264.7 cells using TRIzol (Thermo Fisher Scientific, Waltham, MA, USA). For miRNA quantification, cDNA was synthesized using the TaqMan MicroRNA Reverse Transcription Kit and then subjected to quantitative real-time polymerase chain reaction (qPCR) with TaqMan probes (Applied Biosystems 7300 system; Applied Biosystems, Foster City, CA, USA). The expression of mmu-miR-122, -21, -34a, and -192 was normalized to cel-miR-57 expression (Thermo Fisher Scientific, Waltham, MA, USA).

To evaluate the functional transfer of EVs, RAW264.7 macrophages were incubated in conditioned medium from control or HFFA-treated AML12 hepatocytes for 24 h. Total RNA was isolated from the RAW264.7 cells, reverse-transcribed using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA), and analyzed using SYBR Green-based qPCR on a Bio-Rad Cycler (Bio-Rad). The expression levels of mmu-miR-21, -34a, and -192, as well as the mRNA levels of their target genes, were assessed using *Actb* as the internal reference for normalization. miRNA targets were predicted using

TargetScan and miRanda. All primer sequences are listed in Table 1.

Plasmid construction

The sequences for mouse *Il6st* and miR-192 were retrieved from the National Center for Biotechnology Information (NCBI) database (Accession Nos. NM_010560.3 and NR_029720.1, respectively). The full-length 3'-untranslated region (3'-UTR) of *Il6st*, containing a predicted mmu-miR-192-binding site, was amplified using the SuperScript III One-Step RT-PCR System (Thermo Fisher Scientific, Grand Island, NY, USA). The amplified *Il6st* fragment was verified using agarose gel electrophoresis, purified with the E.Z.N.A Gel Extraction Kit (Omega Bio-tek, Norcross, GA, USA), and cloned into the pmirGLO Dual-Luciferase Vector (Promega, Madison, WI, USA) via NheI and SbfI restriction sites. The amplification primers were as follows: 5'-GGGGCTAGCAGGACTGGCTCCTGAAC-3' and 5'-AACCTGCAGGACACGAAAGAATACGAGCCAC-3'.

Dual-luciferase reporter assay

HEK-293T cells (American Type Culture Collection, Manassas, VA, USA) were co-transfected with the *Il6st* 3'-

UTR luciferase reporter vector and either mmu-miR-192 mimic (200 nM or 250 nM) (GenePharma, Shanghai, China) or a non-targeting control siRNA (TUC339) using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). Luciferase activity was assessed 48 h after transfection using a dual-luciferase assay (Promega), with firefly luciferase signals normalized to *Renilla* luciferase activity.

Recombinant vector with mutant 3'-UTR of *Il6st* for luciferase assay

Wild-type (WT) and mutant *Il6st* 3'-UTR sequences were cloned into the XbaI/XhoI sites of the pmirGLO Dual-Luciferase Vector (Promega) to generate pmirGLO-*Il6st* and pmirGLO-Δ *Il6st*, respectively [19]. Specifically, in the mutant (pmirGLO-Δ *Il6st*) construct, the predicted seed region for mmu-miR-192 was mutated to disrupt miRNA-mRNA base-pairing. The following oligonucleotides were used for construction: pmirGLO-*Il6st* WT: 5'-TCGAGTAGCGGCCGCTATGAAAGTCGGGCACAGGAAGGCCAGGTCAGGGGGCAT-3' and 5'-CTAGATGCCCCTGACCTGGCCTTCTGTGCCGACTTTCATAGCGGCCGCTAC-3'; pmirGLO-Δ *Il6st* MUT: 5'-TCGAGTAGCGGCCGCTATGAAAGTC-

Table 1. qRT-PCR primer and siRNA sequences

Gene	Accession number	Forward sequences	Reverse sequences
<i>Actb</i>	NM_007393.5	5'-GGCTGTATTCCCCTCCATCG-3'	5'-CCAGTTGGTAAACAATGCCATGT-3'
<i>Map4k4</i>	NM_001252200.1	5'-CATCTCCAGGGAAATCCTCAG-3'	5'-TTCTGTAGTCGTAAGTGGCGTCTG-3'
<i>Slpr1</i>	NM_007901.5	5'-GTGTAGACCCAGAGTCCTGCG-3'	5'-AGCTTTTCCTTGCTGGAGAG-3'
<i>Vldlr</i>	NM_001161420.1	5'-GAGCCCCTGAAGGAATGCC-3'	5'-CCTATAACTAGGTCTTTGCAGATATGG-3'
<i>Il17rd</i>	NM_001411765.1	5'-AACAGCGGACTGCACAACAT-3'	5'-GCAAGCGTACTGGCTGATG-3'
<i>Il6st</i>	NM_010560.3	5'-GGAGGAGCGGCCAGAAGAC-3'	5'-AGCCCCGTGCCAAGAG-3'
<i>Il6ra</i>	NM_001310676.1	5'-AAGCAGCAGGCAATGTTACC-3'	5'-CATAAATAGTCCCCAGTGTCG-3'
<i>Tgfb2</i>	NM_009371.3	5'-GTAATAGGACTGCCCATCCAC-3'	5'-GATTTCGTGGTTGTACAGGTG-3'
<i>Stat6</i>	NM_009284.2	5'-TGAGGTGGGGACCAGCCGG-3'	5'-GTGACCAGGACACACAGCGG-3'
<i>Socs4</i>	NM_080843.3	5'-TTCTTCTGGGCATTCTCG-3'	5'-AGACTGATGGCGATGGTGA-3'
<i>Tnfr</i>	NM_001278601.1	5'-GGAGAAGGGTGACCGACTCA-3'	5'-CTGCCCAGACTCGGCAA-3'
<i>Il1b</i>	NM_008361.4	5'-ACAGATGAAGTGCTCCTTCCA-3'	5'-GTCGGAGATTCGTAGCTGGAT-3'
<i>Cxcl10</i>	NM_021274.2	5'-GGCCATCAAGAATTTACTGAAAGCA-3'	5'-TCTGTGTGGTCCATCCTTGGA-3'
<i>Cxcl11</i>	NM_019494.1	5'-CCTTGGCTGTGATATTGTGTGCTA-3'	5'-CCTATGCAAAGACAGCGTCCT-3'
<i>Ccl17</i>	NM_011332.3	5'-TGAGGACTGCTCCAGGGATG-3'	5'-AACGGTGGAGGTCCAGGTA-3'
<i>Nos2</i>	NM_001313921.1	5'-CAGCGGGATGACTTTCCAA-3'	5'-AGGCAAGATTTGGACCTGC-3'
<i>Il12b</i>	NM_001303244.1	5'-TGAAGAAAGATGTTTATGTCTAGTAAT-3'	5'-GGTCCAAGGTCCAGGTGAT-3'
<i>Cd206</i>	NM_008625.2	5'-CGGTGACCTCACAAGTATCCACAC-3'	5'-TTCATCACCACACAATCCTCCTG-3'
<i>Il10</i>	NM_010548.2	5'-GGTTGCCAAGCCTTGCTCTGA-3'	5'-AGGGAGTTCACATGCGCCT-3'
siRNA			
<i>TUC339</i>		5'-GCGACAATTTAGACTCA-3'	

GGGCACAGGAAGGCCTTAAGGGGCAT-3' and 5'-CTAGATGCCCCCTTAAGGCCTTCCTGTGC-CCGACTTTCATAGCGGCCGCTAC-3'.

Plasmids were prepared using the E.Z.N.A. Endotoxin-Free Plasmid DNA Miniprep Kit (TIANGEN, Beijing, China) and verified using sequencing. For the luciferase assay, HEK-293T cells were transfected with 2 µg of each reporter vector and 200 nM miRNA mimics or control siRNA using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA, USA). After 48 h, dual-luciferase activity was measured (Promega), and firefly luciferase levels were normalized to *Renilla* luciferase activity.

***Il6st* level in RAW264.7 cells transfected with mmu-miR-192 mimic**

To investigate the effect of mmu-miR-192 on *Il6st* expression, RAW264.7 cells were transfected with 200 nM mmu-miR-192 mimic or control siRNA. *Il6st* mRNA levels were quantified using SYBR Green-based quantitative reverse transcription PCR (qRT-PCR) on a Bio-Rad Cyclar (Bio-Rad), with *Actb* serving as the internal reference for normalization.

Analysis of mmu-miR-192-regulated macrophage differentiation and polarization

RAW264.7 cells were transfected with mmu-miR-192 mimics or control siRNA or treated with phosphate buffer saline (PBS) (blank control) for 48 h, and then stimulated with interferon-gamma (IFN-γ, 20 ng/ml) (Sigma-Aldrich) and lipopolysaccharide (LPS, 100 ng/ml) (Sigma-Aldrich) for 18 h to induce M1 polarization or with 20 ng/ml IL-4 (PeproTech, Rocky Hill, NJ, USA) to induce M2 polarization. Polarization-specific biomarkers were analyzed using qRT-PCR.

Transfection of pCMV3-*Il6st* vector

pCMV3-*Il6st* and control pCMV3-untagged vectors were obtained from Sino Biological (Beijing, China). RAW264.7 cells were divided into several treatment groups: co-transfection with mmu-miR-192 mimics and pCMV3-*Il6st* or pCMV3-control, transfection with mmu-miR-192 mimics alone, transfection with control siRNA, and blank PBS control. After 48 h, the cells were stimulated to induce M1-associated polarization by incubating with IFN-γ (20 ng/ml) and LPS (100 ng/ml) for 18 h.

qRT-PCR

Total RNA was isolated using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA), and 1 µg of RNA was reverse transcribed to cDNA using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA, USA). Gene expression levels were determined using SYBR Green-based qPCR on a CFX96 system (Bio-Rad,

Hercules, CA, USA) with the primers listed in Table 1. The expression levels of mitogen-activated protein kinase 4 (*Map4k4*) (ID: 26921), sphingosine-1-phosphate receptor 1 (*Slpr1*) (ID:13609), very low-density lipoprotein receptor (*Vldlr*) (ID:22359), interleukin 17 receptor D (*Il17rd*) (ID:171463), interleukin 6 signal transducer (*Il6st*) (ID:16195), interleukin 6 receptor, alpha (*Il6ra*) (ID:16194), transforming growth factor, beta receptor II (*Tgfbr2*) (ID:21813), signal transducer and activator of transcription 6 (*Stat6*) (ID:20852), suppressor of cytokine signaling 4 (*Socs4*) (ID:67296), C-X-C motif chemokine ligand 10 (*Cxcl10*) (ID:15945), motif chemokine ligand (C-X-C motif) ligand 11 (*Cxcl11*) (ID:56066), interleukin 12B (*Il12 p40*) (ID:16160), nitric oxide synthase 2, inducible (*Nos2*) (ID:18126), tumor necrosis factor (*Tnfa*) (ID:21926), interleukin 1 beta (*Il1b*) (ID:16176), interleukin 10 (*Il10*) (ID:16153), C-C motif chemokine ligand 17 (*Ccl17*) (ID:20295), mannose receptor, C type 1 (*Cd206*) (ID:17533), and actin beta (*Actb*) (ID:11461) mRNA expression levels were normalized to the housekeeping gene *Actb* for each sample.

Statistical analyses

Statistical analyses were conducted using IBM SPSS Statistics software (version 22.0; IBM Corp., Armonk, NY, USA) and GraphPad Prism software (version 6; GraphPad Software, La Jolla, CA, USA). All experiments were performed with at least three independent biological replicates. Data are reported as mean ± standard error of the mean. Differences between groups were assessed using one-way analysis of variance; results with a *p*-value of < 0.05 were considered significant.

Results

Macrophages internalized steatotic hepatocyte-derived EVs in an *in vitro* MASLD model

To model MASLD *in vitro*, AML12 hepatocytes were treated with an HFFA mixture for 24 h. Nile Red staining confirmed substantial intracellular lipid accumulation in HFFA-treated cells compared with control cells (Fig. 1A, B). Quantitative analysis revealed a significant increase in the number of lipid droplets (Fig. 1C), thus supporting the successful establishment of the hepatic steatosis model. EVs isolated from HFFA-treated AML12 cells through differential ultracentrifugation exhibited characteristic morphology and size under transmission electron microscopy (Fig. 1D). PKH67-labeled EVs were taken up by RAW264.7 macrophages within 24 h, as visualized using confocal microscopy (Fig. 1E), supporting the possibility of EV-mediated communication between steatotic hepatocytes and macrophages.

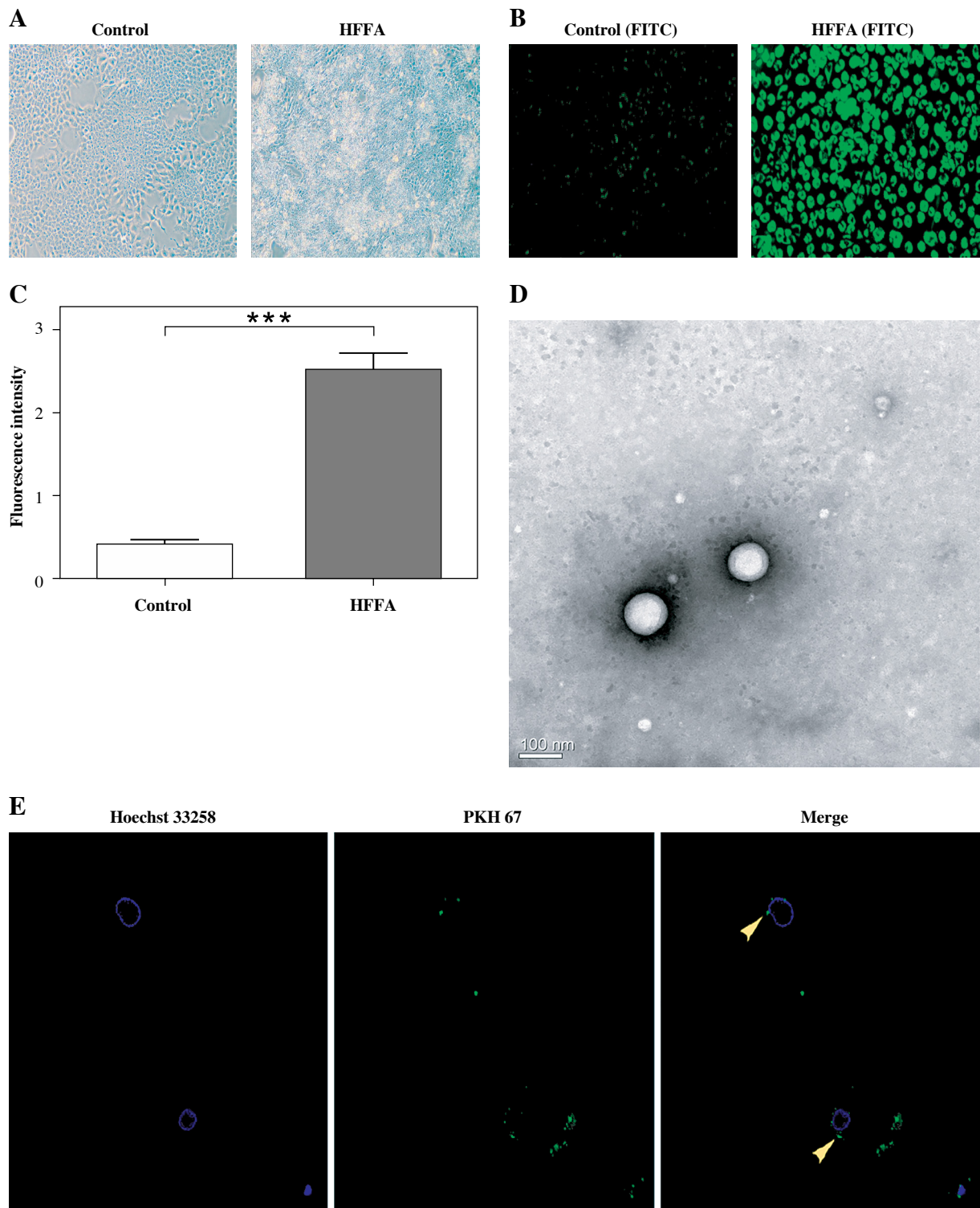


Fig. 1. Extracellular vesicles from steatotic hepatocytes in a metabolic dysfunction-associated steatotic liver disease (MASLD) model are taken up by macrophages. **A, B**) Nile Red staining of AML12 hepatocytes treated with high free fatty acids (HFFAs) or untreated for 24 h, showing increased lipid accumulation (green). **A**) Brightfield images were captured using an Olympus microscope ($\times 10$). **C**) Quantitative analysis of lipid droplet fluorescence. Data are presented as mean $\pm$ SEM ($n = 3$). *** $p < 0.001$. **D**) A representative transmission electron micrograph of isolated extracellular vesicles (EVs). Scale bar: 100 nm. **E**) Confocal microscopy image showing cell-associated fluorescence following exposure to PKH67-labeled EVs (green) from steatotic hepatocytes by RAW264.7 macrophages. The nuclei were stained with Hoechst 33258 (blue). The arrows indicate intracellular extracellular vesicles (EVs)

miRNA enrichment in steatotic hepatocyte-derived EVs upon internalization by macrophages led to significant downregulation of expression of predicted target genes

qPCR analysis revealed significant enrichment of mmu-miR-122, -21, -192, and -34a ($p < 0.05$) in EVs derived from steatotic hepatocytes compared with EVs derived from control hepatocytes (Fig. 2Aa). Correspondingly, RAW264.7 macrophages treated with conditioned medium from steatotic cells showed elevated intracellular levels of these miRNAs ($p < 0.05$, Fig. 2Ab), consistent with miRNA transfer. The expression of bioinformatically predicted target genes for these miRNAs was significantly downregulated in recipient macrophages (Fig. 2B), consistent with post-transcriptional regulation.

Il6st as a target gene of mmu-miR-192

Bioinformatics analysis revealed *Il6st* as a putative target of mmu-miR-192. To validate this interaction, the full-length 3'-UTR of *Il6st* was cloned into a firefly luciferase reporter vector (pmirGLO-*Il6st*; Fig. 3A). Co-transfection of this reporter with mmu-miR-192 mimics, compared with the non-targeting control, significantly reduced luciferase activity in 293T cells (Fig. 3B). No reduction in luciferase activity was observed in cells transfected with control siRNA or empty vector, supporting specific regulation by mmu-miR-192. A potential binding site for mmu-miR-192 was identified within the 3'-UTR of *Il6st*. To test its specificity, a reporter containing the WT binding site and a mutant (MT) version with seed-region substitutions were constructed (Fig. 3C). The WT reporter showed significant downregulation upon mmu-miR-192 mimic transfection, whereas the MT reporter remained unaffected (Fig. 3D), indicating that the predicted site mediates the interaction. Consistent with these findings, overexpression of mmu-miR-192 in RAW264.7 cells reduced *Il6st* mRNA level (Fig. 3E). These results support the conclusion that *Il6st* is a direct target of mmu-miR-192.

mmu-miR-192 promotes M2-associated marker expression and suppresses M1-associated marker expression by targeting *Il6st*

Given the role of IL6ST in macrophage polarization, we investigated the functional consequence of miR-192-mediated IL6ST repression. In IFN- γ /LPS-stimulated RAW264.7 cells, mmu-miR-192 overexpression significantly suppressed the expression of key M1 markers (Fig. 4A-E), and this effect was reversed by co-transfection with an *Il6st* expression vector. Conversely, under IL-4 stimulation, miR-192 upregulated the expression of M2 markers, an effect that was reversed by restoring *Il6st* expression (Fig. 4F-H). These complementary gain-of-function and rescue experiments support a role for *Il6st* in

mediating the effects of mmu-miR-192 on M1- and M2-associated marker expression in macrophages.

Discussion

Our study revealed a potential signaling pathway in MASLD, centered on the transfer of miR-192 via steatotic hepatocyte-derived EVs to macrophages, where it directly targets IL6ST to suppress M1-associated marker expression while promoting M2-associated marker expression. This finding suggests that EV-packaged miR-192 may contribute to the regulation of hepatic immune cell programming within the steatotic microenvironment.

Epidemiologically, MASLD is becoming the leading cause of chronic liver disease worldwide, with prevalence rates varying by ethnicity and region. Recent studies indicate a rising incidence of MASLD in Asia comparable with that in Western countries. While our study focused on the molecular mechanism *in vitro*, understanding the miR-192/IL6ST axis may help identify candidate biomarkers for further investigation of their associations with disease severity in diverse patient populations, although this requires clinical validation.

The role of miRNAs in modulating macrophage polarization is well-established [20], with distinct sets of miRNAs driving either the M1 (e.g., miR-155, miR-9) [21] or M2 (e.g., miR-124, miR-223) [22] phenotype. Our study suggests that miR-192 may also contribute to this regulatory network. Although previous profiling studies have cataloged alterations in the expression of miRNAs in EVs from diseased tissues [23], their functional consequences often remain correlative. By combining a physiologically relevant *in vitro* MASLD model [24] with functional gain- and loss-of-function experiments, we provide experimental evidence supporting a role for miR-192 in regulating macrophage-associated responses. MiR-192 knockdown increased pro-inflammatory cytokine production, phagocytosis, and co-stimulatory molecule expression, whereas its overexpression suppressed the expression of M1-associated markers. The underlying mechanism was investigated using integrated bioinformatics and experimental validation.

Among predicted targets, *Il6st* was confirmed as a direct target of mmu-miR-192 via luciferase reporter and mutational assays. *Il6st* targeting is functionally significant, as the M2-associated marker-expression changes induced by miR-192 was effectively reversed by restoring IL6ST expression. These rescue experiment findings support a role for IL6ST repression in mediating the effects of miR-192. This conclusion aligns with the critical role of IL-6/gp130/STAT3 signaling in macrophage polarization [25] and suggests that EV-associated miR-192 may modulate a major signaling pathway involved in immune responses. Nevertheless, minor contributions from other predicted targets (e.g., *Map4k4* and *Vldlr*) cannot be for-

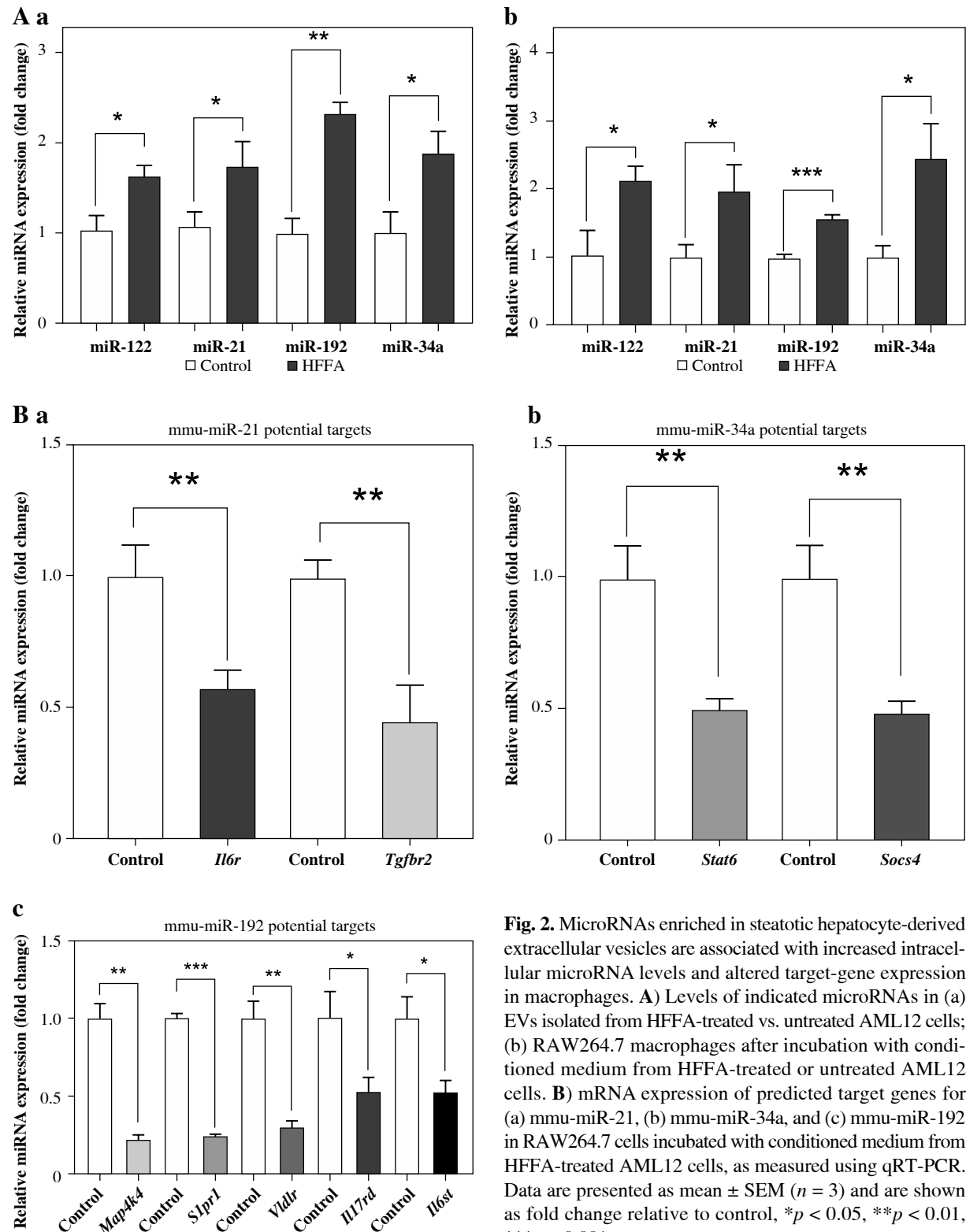


Fig. 2. MicroRNAs enriched in steatotic hepatocyte-derived extracellular vesicles are associated with increased intracellular microRNA levels and altered target-gene expression in macrophages. **A)** Levels of indicated microRNAs in (a) EVs isolated from HFFA-treated vs. untreated AML12 cells; (b) RAW264.7 macrophages after incubation with conditioned medium from HFFA-treated or untreated AML12 cells. **B)** mRNA expression of predicted target genes for (a) mmu-miR-21, (b) mmu-miR-34a, and (c) mmu-miR-192 in RAW264.7 cells incubated with conditioned medium from HFFA-treated AML12 cells, as measured using qRT-PCR. Data are presented as mean $\pm$ SEM ($n = 3$) and are shown as fold change relative to control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

mally excluded, and future combinatorial target modulation studies may further elucidate the target-specific effects of miR-192.

Our results add to evidence for EV-mediated “macrophage education” [26] in metabolic liver disease. Steatotic hepatocytes may influence local inflammation through

EV-associated miR-192, which could represent an adaptive, yet potentially maladaptive, response if it promotes sustained M2-associated responses. Whether this mechanism contributes to disease progression remains to be determined. Although our study reveals a potential pathway, clinical heterogeneity in MASLD is shaped by broader fac-

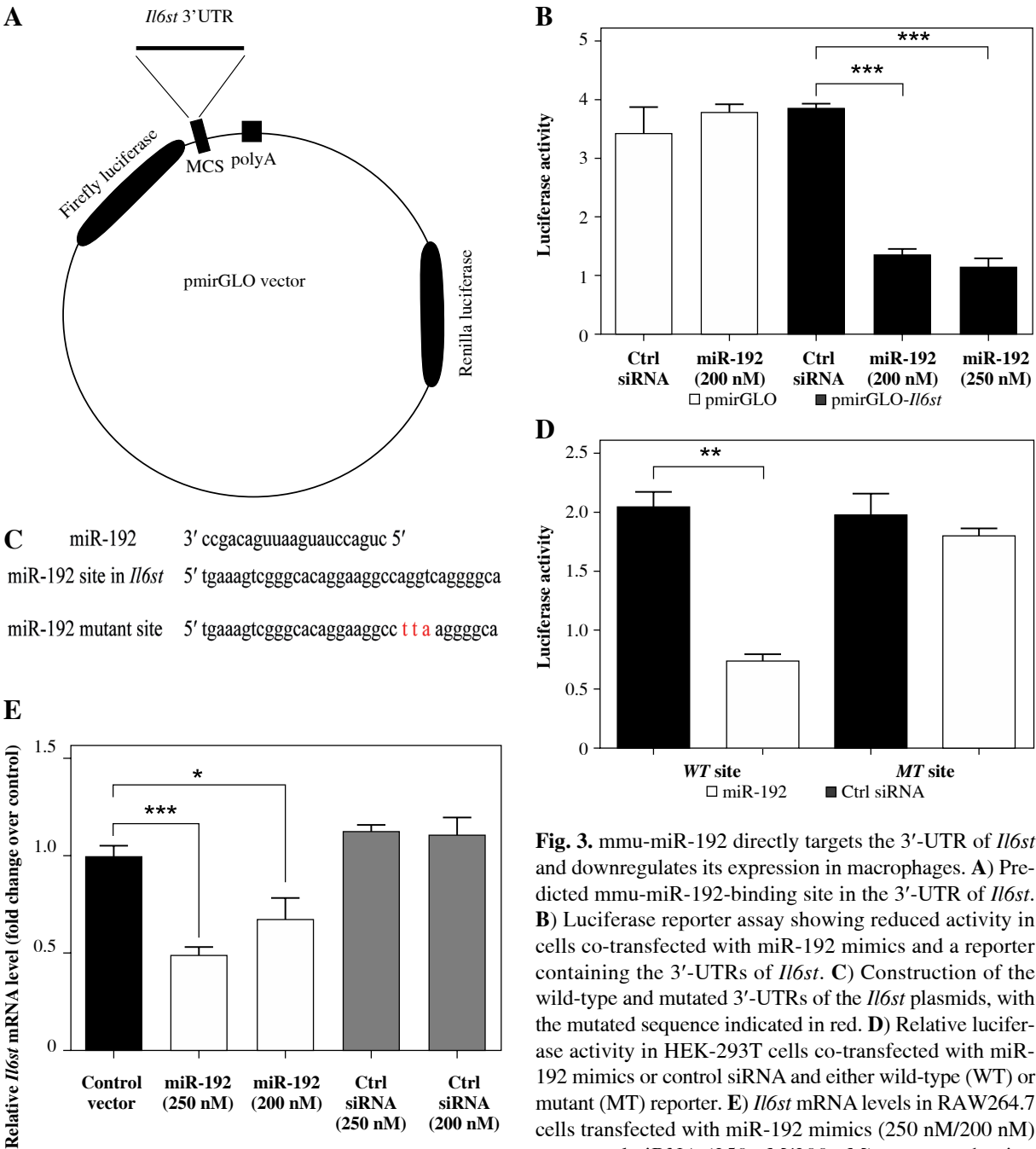


Fig. 3. mmu-miR-192 directly targets the 3'-UTR of *Il6st* and downregulates its expression in macrophages. **A)** Predicted mmu-miR-192-binding site in the 3'-UTR of *Il6st*. **B)** Luciferase reporter assay showing reduced activity in cells co-transfected with miR-192 mimics and a reporter containing the 3'-UTRs of the *Il6st*. **C)** Construction of the wild-type and mutated 3'-UTRs of the *Il6st* plasmids, with the mutated sequence indicated in red. **D)** Relative luciferase activity in HEK-293T cells co-transfected with miR-192 mimics or control siRNA and either wild-type (WT) or mutant (MT) reporter. **E)** *Il6st* mRNA levels in RAW264.7 cells transfected with miR-192 mimics (250 nM/200 nM) or control siRNA (250 nM/200 nM), measured using qRT-PCR. Data are presented as mean $\pm$ SEM ($n = 3$) and are shown as fold change relative to control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

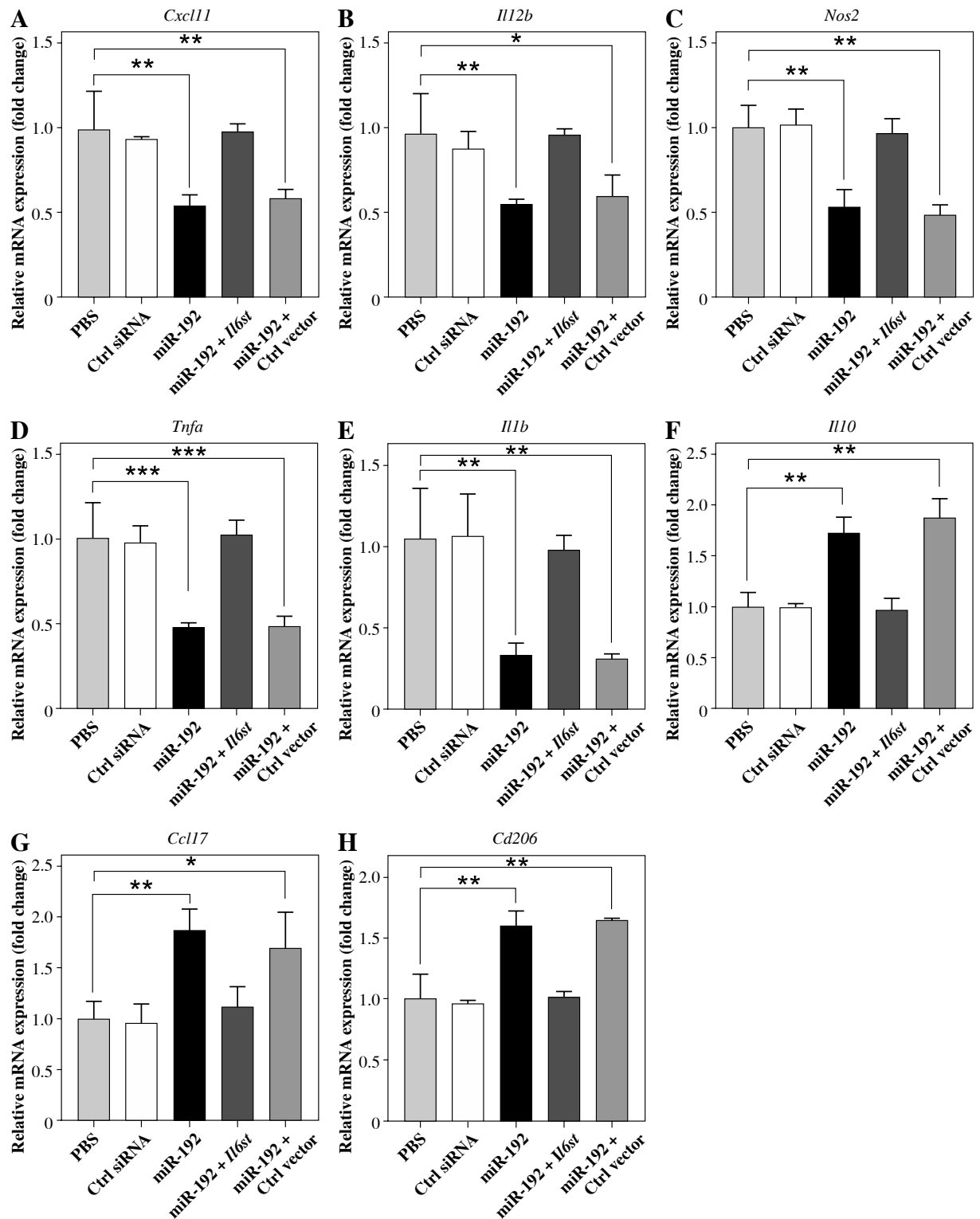


Fig. 4. mmu-miR-192 affects M1- and M2-associated marker expression by targeting *Il6st*. **A-E)** The effects of mmu-miR-192 on M1 polarization were rescued by restoring *Il6st* expression. In IFN- γ /LPS-stimulated macrophages, mmu-miR-192-mediated downregulation of M1 markers (*Cxcl11*, *Il12b*, *Nos2*, *Tnfa*, and *Il1b*) was reversed upon *Il6st* reconstitution. **F-H)** Conversely, under IL-4 stimulation, mmu-miR-192-enhanced expression of M2 markers (*Il10*, *Ccl17*, and *Cd206*) was attenuated by *Il6st* overexpression. Data are presented as mean $\pm$ SEM ($n = 3$) and are shown as fold change relative to control, * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

tors including genetic predisposition, environmental exposures, and ethnic or geographic differences. Additionally, the complexity of the *in vivo* steatotic liver microenvironment should be noted. In MASLD, excessive lipid accumulation is not restricted to hepatocytes; hepatic macrophages also take up lipids and adopt a foamy phenotype, which can intrinsically modulate their activation and polarization and may interact with EV-mediated signaling. Future *in vitro* models should therefore incorporate lipid-loaded macrophages to dissect the interplay between direct lipid exposure and EV-mediated signaling.

Our conclusions are drawn from an *in vitro* co-culture system using the well-established murine AML12 and RAW264.7 cell lines, selected for their experimental tractability, which, despite their utility, cannot fully replicate the complex *in vivo* liver microenvironment or the complete biology of tissue macrophages. Our reductionist *in vitro* system, using naïve RAW264.7 macrophages and synthetic miR-192 mimics, was designed to investigate the contribution of the miR-192/IL6ST axis while minimizing confounding effects from other EV cargo. Consequently, we did not directly use isolated steatotic EVs to assess global M1/M2 shifts under defined polarization conditions nor did we characterize the effects on resting M0 macrophages. Our data therefore indicate that miR-192 primarily modulates the response to polarization stimuli rather than inducing a spontaneous phenotype switch. Polarization was evaluated solely at the transcriptional level using qRT-PCR, without assessing classical surface activation markers such as CD54 and CD86 using flow cytometry. These limitations warrant future investigation.

Furthermore, we focused on the immunoregulatory role of miR-192, but we cannot rule out synergistic or independent effects of other enriched miRNAs in steatotic EVs (e.g., miR-122, -21, -34a). The holistic net effect of the entire EV payload on macrophage phenotypes, and the interplay between direct lipid loading and EV-mediated signaling, remain important areas for future investigation. Ultimately, the translational relevance of the miR-192/IL6ST axis requires validation in clinical cohorts and *in vivo* models. Future studies are needed to determine whether this axis shows differential activity or therapeutic potential across diverse patient populations. Addressing these limitations is essential for translating this mechanistic insight into therapeutic strategies.

In conclusion, we characterized a potential hepatocyte–macrophage communication axis in MASLD. Our findings support a role for miR-192-mediated repression of IL6ST in regulating macrophage responses, although EV-mediated functional transfer requires further confirmation. This axis may contribute to our understanding of parenchymal–immune crosstalk and warrants further investigation as a potential therapeutic target. Strategies aimed at modulating this specific miRNA pathway or harnessing EV biology

for targeted delivery could be explored in future studies to determine whether they can regulate inflammation and alter the progression of MASLD.

Data availability statement

The datasets used or analyzed in this study are available from the corresponding authors on reasonable request.

Acknowledgments

All experiments were performed in the laboratories of the Department of Hematology, Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences), Southern Medical University, Guangdong, China, and the Department of Immunology, West China School of Basic Medical Sciences and Forensic Medicine, Sichuan University, Chengdu, China. This study was supported by Guangdong Provincial People's Hospital (Guangdong Academy of Medical Sciences).

Funding

This research was funded by the Guangdong Provincial Medical Science and Technology Research Foundation of China (No. B2025359).

Disclosures

Approval of the Bioethics Committee was not required. The authors declare no conflict of interest.

References

1. Chan WK, Chuah KH, Rajaram RB, Lim LL, et al. (2023): Metabolic dysfunction-associated steatotic liver disease (MASLD): A state-of-the-art review. *J Obes Metab Syndr* 32: 197-213.
2. Do A, Zahrawi F, Mehal WZ (2025): Therapeutic landscape of metabolic dysfunction-associated steatohepatitis (MASH). *Nat Rev Drug Discov* 24: 171-189.
3. Tincopa MA, Anstee QM, Loomba R (2024): New and emerging treatments for metabolic dysfunction-associated steatohepatitis. *Cell Metab* 36: 912-926.
4. Castera L, Friedrich-Rust M, Loomba R (2019): Noninvasive assessment of liver disease in patients with nonalcoholic fatty liver disease. *Gastroenterology* 156: 1264-1281.e4.
5. European Association for the Study of the Liver (EASL); European Association for the Study of Diabetes (EASD); European Association for the Study of Obesity (EASO) (2024): EASL-EASD-EASO Clinical Practice Guidelines on the management of metabolic dysfunction-associated steatotic liver disease (MASLD). *J Hepatol* 81: 492-542.
6. Abdelhameed F, Kite C, Lagojda L, Dallaway A, et al. (2024): Non-invasive scores and serum biomarkers for fatty liver in the era of metabolic dysfunction-associated steatotic liver disease (MASLD): A comprehensive review from NAFLD to MAFLD and MASLD. *Curr Obes Rep* 13: 510-531.

7. Mily A, Kalsum S, Loreti MG, Rekha RS, et al. (2020): Polarization of M1 and M2 human monocyte-derived cells and analysis with flow cytometry upon *Mycobacterium tuberculosis* infection. *J Vis Exp* 163: 61807.
8. Wang Z, Du K, Jin N, Tang B, et al. (2023): Macrophage in liver fibrosis: identities and mechanisms. *Int Immunopharmacol* 120: 110357.
9. Guo W, Li Z, Anagnostopoulos G, Kong WT, et al. (2024): Notch signaling regulates macrophage-mediated inflammation in metabolic dysfunction-associated steatotic liver disease. *Immunity* 57: 2310-2327.e6.
10. Shaker O, Mahfouz H, Salama A, Medhat E (2020): Long non-coding HULC and miRNA-372 as diagnostic biomarkers in hepatocellular carcinoma. *Rep Biochem Mol Biol* 9: 230-240.
11. Yu J, Peng J, Luan Z, Zheng F, et al. (2019): MicroRNAs as a novel tool in the diagnosis of liver lipid dysregulation and fatty liver disease. *Molecules* 24: 230.
12. Saad El-Din S, Ahmed Rashed L, Eissa M, Eldemery AB, et al. (2022): Potential role of circRNA-HIPK3/microRNA-124a crosstalk in the pathogenesis of rheumatoid arthritis. *Rep Biochem Mol Biol* 10: 527-536.
13. Carpi S, Daniele S, de Almeida JFM, Gabbia D (2024): Recent advances in miRNA-based therapy for MASLD/MASH and MASH-associated HCC. *Int J Mol Sci* 25: 12229.
14. Vishnoi A, Rani S (2023): miRNA biogenesis and regulation of diseases: An updated overview. *Methods Mol Biol* 2595: 1-12.
15. Harrell CR, Jovicic N, Djonov V, Arsenijevic N, et al. (2019): Mesenchymal stem cell-derived exosomes and other extracellular vesicles as new remedies in the therapy of inflammatory diseases. *Cells* 8: 1605.
16. Stefan N, Yki-Järvinen H, Neuschwander-Tetri BA (2025): Metabolic dysfunction-associated steatotic liver disease: heterogeneous pathomechanisms and effectiveness of metabolism-based treatment. *Lancet Diabetes Endocrinol* 13: 134-148.
17. Ye J, Zheng J, Tian X, Xu B, et al. (2022): Fucoxanthin attenuates free fatty acid-induced nonalcoholic fatty liver disease by regulating lipid metabolism/oxidative stress/inflammation via the AMPK/Nrf2/TLR4 signaling pathway. *Mar Drugs* 20: 225.
18. Baxter AA, Phan TK, Hanssen E, Liem M, et al. (2019): Analysis of extracellular vesicles generated from monocytes under conditions of lytic cell death. *Sci Rep* 9: 7538.
19. Zhong Y, Qin C, Wang Q, Ding M, et al. (2024): Inhibition of Foxp3 expression in the placenta of mice infected intraperitoneally by *Toxoplasma gondii* tachyzoites: insights into the PPAR γ /miR-7b-5p/Sp1 signaling pathway. *Parasit Vectors* 17: 189.
20. Liang CL, Jiang H, Feng W, Liu H, et al. (2021): Total glucosides of *pacony* ameliorate pristane-induced lupus nephritis by inducing PD-1 ligands+ macrophages via activating IL-4/STAT6/PD-L2 signaling. *Front Immunol* 12: 683249.
21. Zhang Y, Li X, Wang C, Zhang M, et al. (2020): lncRNA AK085865 promotes macrophage M2 polarization in CVB3-induced VM by regulating ILF2-ILF3 complex-mediated miRNA-192 biogenesis. *Mol Ther Nucleic Acids* 21: 441-451.
22. Essandoh K, Li Y, Huo J, Fan GC (2016): MiRNA-mediated macrophage polarization and its potential role in the regulation of inflammatory response. *Shock* 46: 122-131.
23. Kang H, Liu T, Wang Y, Bai W, et al. (2024): Neutrophil-macrophage communication via extracellular vesicle transfer promotes itaconate accumulation and ameliorates cytokine storm syndrome. *Cell Mol Immunol* 21: 689-706.
24. Abdelgwad M, Zakaria R, Marzouk S, Sabry D, et al. (2023): The emerging role of circular RNA homeodomain interacting protein kinase 3 and circular RNA 0046367 through Wnt/beta-catenin pathway on the pathogenesis of nonalcoholic steatohepatitis in Egyptian patients. *Rep Biochem Mol Biol* 11: 614-625.
25. Liu B, Li J, Chen B, Shuai Y, et al. (2023): Dental pulp stem cells induce anti-inflammatory phenotypic transformation of macrophages to enhance osteogenic potential via IL-6/GP130/STAT3 signaling. *Ann Transl Med* 11: 90.
26. Yao Y, Chen C, Wang J, Xuan H, et al. (2023): Circular RNA circATP9A promotes non-small cell lung cancer progression by interacting with HuR and by promoting extracellular vesicles-mediated macrophage M2 polarization. *J Exp Clin Cancer Res* 42: 330.