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Human umbilical cord mesenchymal stem cell exosomes promote liver regeneration by inducing CD206^{+} macrophage polarization and suppressing ZBP1 PANoptosis

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Abstract

Post-hepatectomy liver failure (PHLF) is a serious complication after partial hepatectomy without approved therapies to safely enhance liver regeneration. This study investigated whether exosomes derived from human umbilical cord mesenchymal stem cells promote liver regeneration by regulating a macrophage-transforming growth factor-beta (TGF-β)-ZBP1-PANoptosis axis. Using a 70% partial hepatectomy mouse model and a macrophage-hepatocyte co-culture system, we found that exosome treatment preserved liver structure, reduced serum injury markers (ALT/AST), increased liver-to-body weight ratio by 32.4%, and elevated proliferating hepatocytes (Ki67-positive cells) by 2.8-fold. Exosomes shifted hepatic macrophages towards a reparative (CD206-positive) phenotype, increased

anti-inflammatory cytokines (interleukin-10 and TGF-β), suppressed ZBP1 expression, and inhibited PANoptosis markers. Overexpression of ZBP1 in macrophages reversed the exosome-induced polarization and cytokine changes. Neutralizing TGF-β blocked the ability of exosome-exposed macrophages to suppress hepatocyte ZBP1 expression and PANoptosis. These results identify a key mechanism by which mesenchymal stem cell-derived exosomes enhance liver regeneration after partial hepatectomy by at least partially promoting TGF-β-dependent reparative macrophage polarization, which inhibits ZBP1-mediated PANoptosis in hepatocytes, suggesting a potential therapeutic strategy for preventing PHLF.

Keywords: Liver regeneration; Mesenchymal stem cell-derived exosomes; Partial hepatectomy; CD206^{+} macrophage; ZBP1; PANoptosis

1 Introduction

Partial hepatectomy (PHx) is the primary curative treatment for primary and secondary liver malignancies, benign liver tumors, and end-stage liver diseases[1]. However, post-hepatectomy liver failure (PHLF) occurs in 8-12% of patients, with a mortality rate exceeding 50% in severe cases[2, 3]. Currently, no approved targeted pharmacological interventions are available to safely accelerate liver regeneration and reduce PHLF risk, representing a major unmet clinical need in hepatobiliary surgery.

The liver has a unique capacity to restore mass and function after tissue loss, a process tightly coordinated by inflammatory cues, cell cycle re-entry, and stromal-parenchymal crosstalk[4]. Among immune cells, hepatic macrophages are pivotal regulators of liver injury and repair[5-7]. Their remarkable plasticity enables

context-dependent transition from pro-inflammatory M1 phenotype to reparative M2 phenotype[8], characterized by CD206 and arginase-1 (Arg1) expression, and secretion of anti-inflammatory cytokines including IL-10 and TGF-β[9]. Accumulating evidence shows that hepatocyte-macrophage crosstalk dynamically controls TGF-β activation across the early and late phases after PHx, directly shaping the tempo of liver regeneration[6, 10].

Mesenchymal stem cell-derived exosomes (MSC-Exo) have emerged as promising cell-free therapeutics for liver diseases[11-13]. In acute and chronic liver injury models, MSC-Exo mitigate inflammation, bias macrophages toward a $CD206^{+}/Arg1^{+}$ phenotype, and accelerate liver repair[14]. Their translational potential is further strengthened by scalable manufacturing, low immunogenicity, and an encouraging clinical safety record from extracellular vesicle clinical trials[15-18]. However, the precise mechanisms by which exosomes reprogram macrophages to protect hepatocytes and sustain liver regeneration remain incompletely defined.

Concurrently, Z-DNA/RNA binding protein 1 (ZBP1) has been identified as a master upstream regulator of PANoptosis, an integrated inflammatory cell death program that simultaneously activates apoptosis, pyroptosis, and necroptosis. ZBP1 senses cytosolic Z-nucleic acids to assemble PANoptosome complexes, which engage caspase-8/-3 (apoptosis), gasdermin D (GSDMD) cleavage (pyroptosis), and RIPK3-MLKL phosphorylation (necroptosis), thereby amplifying inflammatory cell death and tissue injury[19-21]. Relevance to liver regeneration is underscored by the finding that GSDMD-mediated pyroptosis directly suppresses hepatocyte proliferation

and liver regrowth after 70% PHx, implying that constraining PANoptotic tone can remove a key brake on liver restoration[22]. However, the role of ZBP1-mediated PANoptosis in PHx-induced liver regeneration, and its upstream regulation by macrophage-derived cytokines, remain unknown.

To our knowledge, this study is the first to reveal that hUC-MSC-Exo promote liver regeneration via the macrophage-TGF-β-ZBP1-PANoptosis axis. We hypothesized that exosomes induce $CD206^+$ macrophage reparative polarization to secrete TGF-β, which subsequently inhibits hepatocyte ZBP1 expression and PANoptosis, thereby enhancing hepatocyte proliferation and liver regeneration after PHx.

2. Methods

2.1 Animal model and experimental grouping

Male C57BL/6 mice (6-8 weeks old, 20-22 g) were purchased fromJiangsu Wukong Biotechnology Co., Ltd. (Jiangsu, China), and housed in a specific pathogen-free (SPF) environment with constant temperature (22±1℃), humidity (50±5%), 12 h/12 h light-dark cycle, and free access to sterile food and water. Mice were acclimatized for 7 days before surgery. Exosome administration was standardized by protein concentration (100 μg per mouse for in vivo studies), as quantified by BCA assay. Nanoparticle tracking analysis (NTA) revealed a corresponding particle concentration of approximately 1.2×10^{10} particles/mL.

Sample size was determined based on pre-experimental data, with a statistical power of 0.8 and $\alpha=0.05$. Mice were randomly assigned to 3 groups (n=6 per group) using a computer-generated random number table:

1) **Sham group**: Mice underwent laparotomy without liver resection, with 200 μL PBS tail vein injection immediately after surgery.
2) **PHx group**: Mice underwent 70% PHx, with 200 μL PBS tail vein injection immediately after surgery.
3) **PHx+Exo group**: Mice underwent 70% PHx, with 100 μg exosomes (resuspended in 200 μL PBS) tail vein injection immediately after surgery.

The 70% PHx model was established using the standard Higgins-Anderson method: Mice were anesthetized with 2% isoflurane inhalation, the median and left lateral liver lobes were ligated at the hilum with 5-0 silk sutures and resected, with strict intraoperative hemostasis. The abdominal wall and skin were sutured layer by layer. All surgeries were performed by the same surgeon to minimize operational bias. Postoperative analgesia was provided with buprenorphine (0.05 mg/kg, subcutaneous injection) every 12 h for 48 h. The 70% PHx model in mice is a well-established gold-standard platform for studying the acceleration of liver regeneration. Although the mouse liver possesses rapid intrinsic regenerative capacity, this model is widely used to identify positive regulators of regeneration by quantifying early proliferative events, where any intervention that significantly enhances liver mass recovery or hepatocyte proliferation provides meaningful evidence of pro-regenerative efficacy.

Mice were euthanized at 72 h after surgery. Blood samples were collected via cardiac puncture, and serum was separated by centrifugation at 3000×g for 15 min at 4℃. Liver tissues were harvested, weighed, and fixed in 4% paraformaldehyde or frozen at -80℃ for subsequent experiments. All histological and molecular analyses were

performed in a single-blinded manner and the tissue slides were de-identified and randomly coded by an independent investigator, and group identities were only revealed after all quantifications were completed. The detailed information of antibodies and reagents is presented in **Table S1** and **Table S2**.

2.2 Histopathological staining

Liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and sectioned into 4 μm slices.

1) **Hematoxylin and Eosin (H&E) staining:** Sections were deparaffinized, rehydrated, stained with hematoxylin and eosin, and observed under a light microscope to evaluate hepatic architecture and inflammatory infiltration.
2) **Masson's trichrome staining:** Staining was performed according to the manufacturer's protocol to evaluate collagen deposition in liver tissues.

2.3 Immunofluorescence staining

Paraffin sections were deparaffinized, rehydrated, and subjected to antigen retrieval using EDTA buffer (pH 9.0) in a microwave. After washing with PBS, endogenous peroxidase activity was blocked with 3% H_2O_2 in the dark for 15 min. Sections were blocked with 10% normal goat serum for 1 h at room temperature, then incubated overnight at 4°C with primary antibodies against CD206 (1:200), CD86 (1:200).

After washing, sections were incubated with HRP-conjugated secondary antibodies in the dark for 1 h, then sequentially treated with tyramine-CY3 and tyramine-488 reagents. A second round of antigen retrieval using citrate buffer (pH 6.0) was performed between double staining steps. Nuclei were counterstained with DAPI.

Slides were mounted with anti-fade mounting medium and imaged under a fluorescence microscope (Olympus, Japan). For quantitative analysis, 5 random high-power fields (HPF, 400×) were selected per section, and the positive cell ratio was calculated using ImageJ software (NIH, USA)

2.4 Immunohistochemistry (IHC) staining

Paraffin sections were deparaffinized, rehydrated, and subjected to antigen retrieval. Endogenous peroxidase activity was blocked with 3% H_2O_2, and sections were blocked with 10% goat serum for 1 h. Sections were incubated with primary antibody against Ki67 (1:200) overnight at 4℃, then incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Signals were developed using DAB substrate, and nuclei were counterstained with hematoxylin. The number of Ki67-positive cells was counted in 5 random HPFs per section[23, 24].

2.5 Western blot assay

Total proteins were extracted from liver tissues or cells using RIPA lysis buffer containing protease and phosphatase inhibitors, on ice for 30 min. Lysates were centrifuged at 12,000 rpm for 10 min at 4℃, and the supernatant was collected. Protein concentration was determined using the BCA method.

Equal amounts of protein were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to polyvinylidene fluoride (PVDF) membranes (Merck Millipore, USA). Membranes were blocked with 5% non-fat milk for 1 h at room temperature, then incubated overnight at 4℃ with primary antibodies against: ZBP1, cleaved caspase-3, cleaved GSDMD, p-MLKL, Arg1, Cyclin D1, and

GAPDH (all 1:1000).

After washing, membranes were incubated with HRP-conjugated secondary antibodies (1:5000) for 1 h at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) kit, and gray value analysis was performed using ImageJ software. GAPDH was used as the internal reference[25, 26].

2.6 Quantitative real-time PCR (qPCR)

Total RNA was extracted from liver tissues or cells using Trizol reagent, and reverse transqPCR was performed using SYBR Green Master Mix on a StepOnePlus Real-Time PCR System (Applied Biosystems, USA). The relative mRNA expression was calculated using the 2^(-ΔΔCt) method, with GAPDH as the internal reference.

2.7 Cell culture and treatment

RAW264.7 mouse macrophage cell line and AML12 mouse hepatocyte cell line were purchased from the American Type Culture Collection (ATCC, USA). RAW264.7 cells were cultured in DMEM medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin. AML12 cells were cultured in DMEM/F12 medium supplemented with 10% FBS, 1× ITS supplement, 40 ng/mL dexamethasone, 100 U/mL penicillin, and 100 μg/mL streptomycin. All cells were cultured at 37℃ with 5% CO_2, and confirmed to be mycoplasma-free.

2.7.1 ZBP1 overexpression in RAW264.7 macrophages

ZBP1 overexpression plasmid (pcDNA3.1-ZBP1) and negative control plasmid (pcDNA3.1-NC) were constructed by Gene Synthesis Company. RAW264.7 cells were transfected with plasmids using Lipofectamine 3000 reagent according to the

manufacturer's protocol. Transfection efficiency was verified by Western blot and qPCR at 48 h after transfection.

2.7.2 Macrophage-hepatocyte co-culture system

A Transwell co-culture system (0.4 μm pore size, Corning, USA) was used to establish non-contact co-culture of RAW264.7 macrophages and AML12 hepatocytes. Briefly, RAW264.7 cells were seeded in the upper chamber, pretreated with or without 100 μg/mL exosomes for 24 h, with or without ZBP1 overexpression. AML12 cells were seeded in the lower chamber and cultured for 24 h. For TGF-β neutralization experiments, 10 μg/mL TGF-β neutralizing antibody or isotype control antibody was added to the upper chamber during exosome treatment.

2.8 EdU proliferation assay

AML12 cell proliferation was detected using a Cell-Light EdU Apollo567 In Vitro Kit according to the manufacturer's protocol[27, 28]. EdU-positive cells were counted in 5 random HPFs per well, and the proliferation rate was calculated.

2.9 Enzyme-linked immunosorbent assay (ELISA)

Serum and hepatic homogenate levels of IL-1β, TNF-α, IL-10, and TGF-β, as well as cell culture supernatant levels of IL-10 and TGF-β, were measured using commercial ELISA kits according to the manufacturer's protocol.

2.10 Statistical analysis

All statistical analyses were performed using GraphPad Prism 9.0 software. Data are presented as mean ± standard deviation (SD) from at least 3 independent biological replicates. All datasets used for parametric testing conformed to a normal distribution

as assessed by the Shapiro-Wilk test ($p > 0.05$). For comparisons between two groups, the unpaired Student's t-test was used. For comparisons among multiple groups, one-way analysis of variance (ANOVA) was used, followed by Tukey's post-hoc test for multiple comparisons. A two-tailed P-value < 0.05 was considered statistically significant. The 72-hour time point was selected for analysis because it represents the critical early phase of liver regeneration, corresponding to the peak of hepatic macrophage polarization and the initiation of hepatocyte cell cycle re-entry.

3 Results

3.1 Exosomes promote postoperative liver regeneration and improve tissue structure

H&E staining showed that at 72 h post-PHx, the PHx group exhibited mild, transient physiological injury responses, including mild hepatic cord disorganization, focal inflammatory infiltration, and hepatocellular edema, while the PHx+Exo group showed relatively intact structure and reduced inflammation (**Fig. 1A**). Masson's trichrome staining revealed significant periportal collagen deposition in the PHx group compared with the Sham group (***P<0.001). This observation reflects transient extracellular matrix (ECM) remodeling during the early phase of liver regeneration, which may provide temporary structural support for proliferating hepatocytes and sinusoidal reconstruction, rather than representing pathological fibrosis as seen in chronic liver disease. Exosome treatment significantly attenuated this collagen accumulation, as evidenced by a marked reduction in collagen fiber percentage in the PHx+Exo group compared with the PHx group (**Fig. 1B**, **P<0.01).

In the PHx model, the PHx group displayed significantly decreased liver-to-body weight ratio and reduced liver volume compared with the Sham group (***P<0.001), indicating impaired liver function. However, after exosome intervention, both the liver-to-body weight ratio and liver volume were significantly restored in the PHx+Exo group compared with the PHx group (**Fig. 1C**, *P < 0.05, **P < 0.01), suggesting that exosomes promote postoperative liver regeneration.

3.2 Exosomes induce M2-type macrophage polarization

Immunofluorescence staining of liver tissue sections from Sham, PHx, and PHx+Exo groups was performed to assess macrophage infiltration and polarization. As shown in the representative images (**Fig. 2**), minimal $CD68^+$ and $CD206^+$ signals were detected in the Sham group. In the PHx group, $CD206^+$ macrophage infiltration was markedly increased, while $CD68^+$ signal remained relatively low. Notably, in the PHx+Exo group, exosome intervention not only further increased $CD68^+$ macrophage accumulation but also substantially enhanced $CD206^+$ fluorescence intensity, indicating a shift toward M2-type polarization.

Furthermore, quantitative analysis of fluorescence intensity showed that CD206 expression was significantly upregulated in the PHx+Exo group compared with the PHx group (***P<0.001), while CD68 expression was also increased but to a lesser extent(*P < 0.05), resulting in a higher CD206/CD68 polarization index (**Fig. 2**). These data confirm that exosomes promote the polarization of macrophages toward an anti-inflammatory, tissue-repairing M2 phenotype in postoperative liver tissue.

3.3 Exosomes reduce ZBP1 expression and inhibit hepatocyte cell death signaling

pathways

Western blot analysis was performed to assess ZBP1 expression and downstream cell death signaling pathways in liver tissues of Sham, PHx, and PHx+Exo groups. Quantitative analysis showed these observations (**Fig. 3A**), showing that ZBP1 expression was significantly elevated in the PHx group compared with the Sham group (***P<0.001), while exosome treatment significantly reduced ZBP1 expression compared with the PHx group (***P<0.001). Similarly, immunohistochemical staining of ZBP1 (**Fig. 3B**) further validated that ZBP1 intensity was strongly increased in the PHx group, whereas exosome intervention markedly attenuated this increase (*P < 0.05, **P < 0.01).

Furthermore, Western blot analysis of PANoptosis-associated executioner proteins was performed (**Fig. 3C**). The protein levels of cleaved caspase-3 (apoptosis), cleaved GSDMD (pyroptosis), and p-MLKL (necroptosis) were all markedly elevated in the PHx group compared with the Sham group. Notably, exosome treatment significantly reduced the levels of all three proteins. Quantitative analysis (**Fig. 3C**, right panels) confirmed that cleaved caspase-3, cleaved GSDMD, and p-MLKL levels were significantly decreased in the PHx+Exo group compared with the PHx group (all ***P<0.001). These results demonstrate that exosomes inhibit ZBP1-mediated activation of multiple cell death signaling pathways following PHx.

3.4 Exosomes enhance hepatocyte proliferation and improve the immune microenvironment

Immunohistochemical staining of Ki67 was performed to assess hepatocyte

proliferation in liver tissues of Sham, PHx, and PHx+Exo groups. As shown in **Fig. 4A** (left panel) , Ki67-positive cells were rarely observed in the Sham group, markedly increased in the PHx group, and further increased in the PHx+Exo group. Quantitative analysis (**Fig. 4A**, right panel) revealed that the proportion of $Ki67^+$ hepatocytes was 22.6% in the PHx group and 33.6% in the PHx+Exo group, representing a 1.5-fold increase (**P<0.01), demonstrating that exosomes promote hepatocyte proliferation.

ELISA was performed to measure cytokine levels in serum. As shown in **Fig. 4B,** compared with the Sham group, the PHx group showed significantly elevated levels of the pro-inflammatory cytokines TNF-α and IL-1β (**P<0.01, ***P<0.001), as well as significantly increased secretion of the anti-inflammatory cytokines IL-10 and TGF-β1 (*P < 0.05, ***P<0.001). After exosome intervention (PHx+Exo group), IL-10 and TGF-β1 levels were further elevated compared with the PHx group (both **P<0.01), while TNF-α and IL-1β levels were significantly reduced (**P<0.01, ***P<0.001). In hepatic homogenate (**Fig.4C**): IL-10 and TGF-β exhibited no statistical differences among three groups (ns). PHx significantly upregulated TNF-α and IL-1β versus Sham (both ***P<0.001), and exosome treatment markedly decreased these two pro-inflammatory cytokines in PHx+Exo (both ***P<0.001).These results confirm that exosomes improve the liver regenerative microenvironment by modulating the balance of pro-inflammatory and anti-inflammatory cytokines.

3.5 Exosomes mediate macrophage polarization and promote hepatocyte

regeneration via ZBP1 regulation

To investigate whether exosomes regulate macrophage polarization via ZBP1, RAW264.7 macrophages were treated with exosomes, with or without ZBP1 overexpression. As shown in **Fig. 5A-D**, exosome treatment significantly upregulated the M2 marker CD206 detected by flow cytometry (Fig.5A), alongside elevated Arg1 mRNA and protein abundance (**Fig.5B-C**) and boosted secretion of anti-inflammatory IL-10 and TGF-β (**Fig.5D**, ***P<0.001). Conversely, macrophage ZBP1 overexpression reversed these exosome-induced phenotypic shifts. These data confirm exosomes drive M2 macrophage polarization by suppressing ZBP1.

A RAW264.7-AML12 co-culture model was further used to verify the paracrine hepatoprotective effect of exosome-primed macrophages. In the co-culture system, exosome preconditioning robustly suppressed proteins associated with multiple cell death pathways (cleaved caspase-3, cleaved GSDMD, p-MLKL) in AML12 hepatocytes (**Fig.**5E, *P<0.05, ***P<0.001). In contrast, macrophage ZBP1 overexpression fully abolished such anti-PANoptosis protection and restored high levels of the three cell-death proteins (*P<0.05, **P<0.01 or ***P<0.001). Collectively, exosomes restrain hepatocyte PANoptosis in a macrophage ZBP1-dependent manner.

EdU proliferation assay and Cyclin D1 immunoblot were applied to detect hepatocyte proliferative capacity. The EdU-positive proportion and Cyclin D1 protein level were significantly elevated after exosome intervention in co-culture (**Fig.5F-G**, **P<0.01, ***P<0.001). However, ZBP1 overexpression in macrophages remarkably blunted

exosome-mediated hepatocyte proliferation, decreasing EdU ratio (***P<0.001) and Cyclin D1 abundance (*P<0.05). These results support that exosome-educated macrophages facilitate hepatocyte proliferation via repressing macrophage ZBP1.

3.6 TGF-β is a key factor in macrophage-mediated hepatocyte protection

Western blot analysis was performed to assess ZBP1 expression and downstream PANoptosis executioner proteins in hepatocytes treated with control medium, exosome-conditioned medium (Exo-CM), and Exo-CM with TGF-β neutralizing antibody (Exo-CM + anti-TGF-β). As shown in the representative blots (**Fig. 6A**, left panel), Exo-CM treatment significantly downregulated ZBP1 protein expression compared with the control group, while addition of TGF-β neutralizing antibody reversed this effect. Quantitative analysis (**Fig. 6A**, right panel) confirmed that ZBP1 expression was significantly reduced in the Exo-CM group compared with the control group (***P<0.001), and this reduction was partially reversed by TGF-β neutralization (***P<0.001).

Similarly, Exo-CM treatment significantly reduced the levels of cleaved caspase-3, cleaved GSDMD, and p-MLKL compared with the control group, whereas TGF-β neutralization restored their expression (**Fig. 6B**,*P < 0.05, **P < 0.01, ***P<0.001).

EdU staining was performed to assess hepatocyte proliferation (**Fig. 6C**). The EdU-positive cell ratio was markedly increased in the Exo-CM group compared with the control group (***P<0.001), indicating that the conditioned medium contains effective pro-proliferative factors. However, this effect was significantly weakened by TGF-β neutralization (**P<0.01).

ELISA further confirmed that TGF-β levels in Exo-CM were significantly elevated compared with the control group (**P<0.01), and the TGF-β neutralizing antibody reduced them to baseline levels (**Fig. 6D**, **P<0.01). These results demonstrate that TGF-β is a key effector molecule through which Exo-CM exerts its hepatoprotective and pro-proliferative functions.

4 Discussion

This study demonstrates that hUC-MSC-derived exosomes orchestrate a two-pronged pro-regenerative program after PHx: they reprogram hepatic macrophages toward a $CD206^{+}/Arg1^{+}$, IL-10/TGF-β-rich reparative phenotype, and concurrently dampen hepatocyte ZBP1 signaling via macrophage-secreted TGF-β, thereby inhibiting PANoptosis and licensing hepatocyte cell cycle re-entry. To our knowledge, this is the first study to identify the macrophage-TGF-β-ZBP1-PANoptosis axis as a key regulatory mechanism of exosome-mediated liver regeneration, providing a novel mechanistic insight and therapeutic target for preventing PHLF.

4.1 Exosomes as immunoreprogramming therapeutics in liver repair

Beyond serving as disease biomarkers, exosomes are active biological therapeutics whose cargo can reset macrophage phenotype and cytokine output. Prior studies have shown that MSC-derived exosomes mitigate liver injury, promote M2 macrophage polarization, and accelerate liver regeneration in PHx models[14]. However, the downstream effector molecules and the direct link between macrophage reprogramming and hepatocyte protection remain unclear. Our study extends these findings by demonstrating that exosome-educated $CD206^{+}$ macrophages secrete

TGF-β, which directly inhibits hepatocyte ZBP1 expression and PANoptosis, forming a complete regulatory axis between immune cells and parenchymal cells. This finding expands the current understanding of exosome-mediated immunomodulation, shifting the focus from single-cell phenotype regulation to multi-dimensional immune-hepatocyte crosstalk.

It is worth noting that TGF-β exhibits well-documented dual roles in liver disease, which has long been a focus of research controversy. TGF-β's effects are highly dependent on the cellular source, time window, and local microenvironment. In chronic liver injury, TGF-β derived from hepatic stellate cells is the key driver of liver fibrosis[29, 30]. In contrast, in the early phase after PHx, macrophage-derived TGF-β primarily promotes hepatocyte proliferation and liver regeneration, with minimal pro-fibrotic effect[10]. In this study, exosome-induced TGF-β was exclusively derived from M2 macrophages, and the intervention was administered immediately after PHx (early regenerative phase). Consistent with previous reports[31], we observed that exosome treatment attenuated, rather than enhanced, collagen deposition in liver tissues, confirming that the macrophage-derived TGF-β pulse in the early postoperative period exerts a pro-regenerative effect rather than pro-fibrotic activity.

Our findings are consistent with and extend a previous study by Elchaninov et al., who demonstrated that bone marrow MSC-derived exosomes promote $CD206^{+}$ macrophage polarization and liver regeneration in an 80% subtotal hepatectomy rat model[32]. This prior work provides important cross-species validation of our core observations in the mouse 70% PHx model. However, several key advances

distinguish our study. First, Elchaninov et al. used an 80% subtotal hepatectomy model mimicking post-transplant small-for-size syndrome, whereas our 70% PHx model better reflects conventional liver resection. Second, while Elchaninov et al. established the role of exosomes in M2 polarization, the downstream effector mechanism remained undefined; our study identifies the complete TGF-β-ZBP1-PANoptosis axis as a key mechanistic link. Third, our study includes rigorous MISEV2018-compliant exosome characterization, co-culture systems, and ZBP1 gain-of-function validation not addressed in the prior work.

4.2 ZBP1 as a druggable upstream governor of PANoptosis in liver regeneration

ZBP1 has emerged as a master regulator of PANoptosis[33], integrating danger sensing with the simultaneous activation of three major inflammatory cell death pathways[34]. Previous studies have shown that GSDMD-mediated pyroptosis suppresses liver regeneration after PHx[22], but the role of ZBP1-mediated PANoptosis in liver regeneration remains unknown. Our study is the first to demonstrate that ZBP1 expression is markedly upregulated in the liver after PHx, and that exosome treatment significantly downregulates ZBP1 and simultaneously inhibits apoptosis, pyroptosis, and necroptosis in hepatocytes. Forced ZBP1 overexpression in macrophages completely reversed the protective and pro-regenerative effects of exosomes, placing ZBP1 as the central upstream node of the cell death restraint that enables liver regeneration.

This finding has important translational implications: modulating a single target (ZBP1) can simultaneously relax three distinct cell death modalities, which is more

effective than targeting a single cell death pathway. Given that multiple forms of cell death are activated in the liver after PHx, ZBP1 represents a promising druggable target for developing therapies to accelerate liver regeneration.

We wish to clarify the interpretation of PANoptosis in our study. Detecting concurrent upregulation of cleaved caspase-3 (apoptosis), cleaved GSDMD (pyroptosis), and p-MLKL (necroptosis) alone does not definitively establish PANoptosome complex formation. Our data demonstrate ZBP1-dependent simultaneous activation of these three pathways, which is consistent with PANoptosis-like cell death but not formal proof of canonical PANoptosis. Therefore, we interpret our findings as evidence of coordinated activation of multiple cell death pathways and use terms such as “PANoptosis-associated markers” and “features consistent with PANoptosis” throughout the manuscript.

4.3 Clinical translation potential

Exosomes have several advantages for clinical translation: low immunogenicity, scalable manufacturing, long-term storage at -80℃, and a favorable safety profile in completed clinical trials[35]. Compared with live MSC therapy, exosome-based treatment avoids the risk of tumorigenesis and immune rejection associated with cell transplantation, which is a major advantage for perioperative clinical application[36].

However, it is important to emphasize that all findings in this study are derived from a mouse model of 70% PHx, and clinical application remains distant. Based on our preclinical results, we speculate that exosome therapy might be further investigated in conditions such as PHx for hepatocellular carcinoma or small-for-size liver syndrome,

but these remain hypothetical at this stage. We wish to stress that extensive validation in large animal models and subsequent clinical trials is mandatory before any clinical translation can be considered.

A concern regarding the use of human exosomes in mouse models is potential xenogeneic immunogenicity. Exosomes are acellular nanovesicles that lack MHC complexes and co-stimulatory molecules, which minimizes the risk of T-cell activation[37]. Numerous studies have successfully used human MSC-exosomes in immunocompetent mice with consistent efficacy and no reported immune rejection[38, 39]. While a subtle immune response cannot be formally excluded, the current evidence supports their safety and bioactivity in mouse models.

We also wish to clarify the distinction between Ki67 expression and mitotic activity. Ki67 is expressed throughout the active cell cycle (G1, S, G2, and M phases) but is absent in quiescent cells. Its increased expression at 72 h post-PHx reflects hepatocyte entry into the cell cycle, which precedes the appearance of visible mitotic figures. Regarding the increased liver weight observed at 72 h post-PHx, we propose that this early mass restoration is the synergistic result of multiple factors: 1) early hepatocyte hypertrophy, 2) Ki67-labeled cell cycle activation (rather than mitosis alone), and 3) reduced hepatocyte loss through exosome-mediated inhibition of ZBP1-dependent PANoptosis. Therefore, our data support that exosomes accelerate the initiation of hepatocyte proliferation rather than completion of mitosis at this early time point.

4.4 Limitations and future directions

Our study has several limitations that need to be addressed in future research. First,

we only verified the role of TGF-β in the regulatory axis, while the contribution of IL-10, another key anti-inflammatory cytokine secreted by M2 macrophages, remains unclear. Subsequent studies will use IL-10 neutralization antibodies and IL-10 knockout macrophage models to explore whether IL-10 plays a synergistic or independent role in this regulatory axis. Second, we did not identify the specific cargo in exosomes that drives macrophage M2 polarization and ZBP1 inhibition. Future work will combine small RNA sequencing and proteomic profiling of exosomes to screen candidate miRNAs, lncRNAs, or proteins, followed by gain- and loss-of-function experiments to validate their specificity and sufficiency. Third, our study was performed only in male mice, and the effect of sex differences on liver regeneration and exosome therapeutic efficacy needs to be validated in female mice. Fourth, the results were obtained from a mouse model, and further validation in large animal models (such as miniature pigs) with liver anatomy and physiology similar to humans is required before clinical translation. Fifth, the pro-fibrotic effect of TGF-β in chronic liver disease should be noted; future studies will explore targeted delivery of exosomes to hepatic macrophages via surface modification to avoid potential off-target effects. Sixth, while our data demonstrate that the TGF-β/ZBP1 axis plays a critical role in exosome-mediated liver regeneration, we do not claim this is the sole mechanism. Exosomes carry a complex cargo of miRNAs, proteins, and lipids, and it is highly likely that multiple pathways act synergistically to produce the observed regenerative effects. The present study focused on the macrophage-TGF-β-ZBP1-PANoptosis axis as one key mechanism; identifying

additional effectors (e.g., exosomal miR-182-5p, miR-21-5p, or other cytokines such as IL-10) and delineating their interactions with this axis are important priorities for future research.

Conclusions

In conclusion, our study demonstrates that hUC-MSC-derived exosomes promote liver regeneration after PHx via two coordinated mechanisms: inducing $CD206^+$ macrophage reparative polarization to secrete TGF-β, and subsequently inhibiting hepatocyte ZBP1-mediated activation of multiple cell death pathways (features consistent with PANoptosis) to enhance hepatocyte proliferation. This study identifies a novel macrophage-TGF-β-ZBP1-PANoptosis regulatory axis, providing a promising cell-free biological therapeutic strategy to prevent PHLF and accelerate liver regeneration after hepatectomy.

Abbreviations

PHx: Partial hepatectomy

PHLF: Post-hepatectomy liver failure

hUC-MSC: Human umbilical cord mesenchymal stem cell

Exo: Exosomes

ZBP1: Z-DNA/RNA binding protein 1

TGF-β: Transforming growth factor beta

IL-10: Interleukin 10

Arg1: Arginase-1

PANoptosis: Inflammatory programmed cell death integrating apoptosis, pyroptosis,

and necroptosis

GSDMD: Gasdermin D

MLKL: Mixed lineage kinase domain-like protein

TEM: Transmission electron microscopy

NTA: Nanoparticle tracking analysis

SDS-PAGE: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

PVDF: Polyvinylidene fluoride

qPCR: Quantitative real-time PCR

IHC: Immunohistochemistry

H&E: Hematoxylin and eosin

ELISA: Enzyme-linked immunosorbent assay

ALT: Alanine transaminase

AST: Aspartate transaminase

TBIL: Total bilirubin

ALB: Albumin

ANOVA: Analysis of variance

SD: Standard deviation

Declarations

Consent for publication

All authors read the manuscript fully and consented to its publication.

Availability of data and materials

The datasets generated during the current study were available from the corresponding author on reasonable request.

Author contributions

Xiaojing Song: Conceptualization, Methodology, Investigation, Writing – Original Draft. Xiao Tang: Methodology, Investigation, Writing – Review & Editing. Yao Liu: Data Curation, Investigation, Writing – Review & Editing. Songtian Liu: Formal analysis, Writing – Review & Editing. Jian Ma: Methodology, Investigation, Writing – Review & Editing. Yi Shao: Conceptualization, Supervision, Validation, Writing – Review & Editing.

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Competing interests

The authors declare that they have no conflicts of interest with the contents of this article.

Ethics approval and consent to participate

All animal experiments were approved by the Animal Experiment Ethics Committee of Bestcell Model Biological Center. These experiments were in compliance with the "Detailed Rules for the Administration of Medical Laboratory Animals" issued by the

Ministry of Health and the "Regulations on the Administration of Laboratory Animals" issued by the National Science and Technology Commission.

Supplementary Material

Table S1: Primer sequences in this study.

Table S2: Key Resource Table.

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Figure Legends

Fig.1 Exosomes Promote Liver Regeneration and Reduce Fibrosis Post-PHx. (A) Representative liver images 7 days post-PHx. Sham: no surgery, PHx: surgery, PHx+Exo: surgery + exosome treatment. (B) Histology (H&E, top) and fibrosis assessment (Masson's trichrome staining, bottom; Scale bar shown) 7d post-PHx. Quantitative analysis shows significantly reduced collagen area in PHx+Exo vs PHx (***P < 0.001 vs Sham; **P < 0.01 vs PHx; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3). (C) Liver regeneration assessed by liver-to-body weight ratio and liver volume 7d post-PHx. PHx+Exo significantly increased both ratios compared to PHx (*P < 0.05, **P < 0.01 vs PHx; ***P < 0.001 vs Sham; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons , n=6).

Fig. 2. Exosomes Modulate Macrophage Polarization in Regenerating Liver. Immunofluorescence (IF) staining for macrophage markers in liver tissue 7d post-PHx (nuclei: DAPI, blue; M2 marker: CD206, green; pan-macrophage marker: CD68, red; Merge; Scale bar shown). (A) Representative images. (B) Quantitative analysis shows mean CD206 fluorescence intensity (M2 marker) decreased in PHx and partially recovered with Exo. Mean CD68 intensity (total macrophages) increased in PHx and Exo attenuated this (*P < 0.05, **P < 0.01 vs Sham; *P < 0.05, ***P < 0.001 vs PHx; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3).

Fig.3 Exosomes target ZBP1-induced cell death during regeneration. (A) Western blot (left) and quantification (right) of ZBP1 protein in liver lysates 7d post-PHx. Exosomes significantly reduced elevated ZBP1 levels (***P < 0.001 vs Sham and vs PHx; one-way ANOVA, n=3). (B) Representative IHC staining for ZBP1 (Scale bar shown) and quantitative analysis of mean optical density. PHx increased ZBP1; Exo attenuated this increase (**P < 0.01 vs Sham; *P < 0.05 vs PHx; one-way ANOVA, n=3). (C) Western blot (left) and quantification (right panels) of panoptosis markers: Cleaved-caspase3 (apoptosis), Cleaved-GSDMD (pyroptosis), p-MLKL (necroptosis). Exosomes significantly reduced levels elevated by PHx (***P < 0.001 vs Sham and vs PHx; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3).

Fig.4 Exosomes Enhance Hepatocyte Proliferation and Modulate Regeneration-Associated Cytokines. (A) Hepatocyte proliferation. Left: Representative Ki67 IHC staining (proliferation marker) in liver tissue 7d post-PHx (Scale bar shown). Right: Quantitative Ki67-positive cell ratio. Exosomes significantly increased proliferation vs PHx (**P < 0.01 vs PHx; **P < 0.01 vs Sham; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3). (B) Levels of key immune cytokines measured in serum or tissue. Exosomes significantly altered levels of TNF-α, IL-1β, IL-6, IL-10, and IL-4 associated with PHx-induced inflammation towards a pro-regenerative profile (Sham comparisons: *P < 0.05, **P<0.01, ***P<0.001; PHx comparisons: **P<0.01, ***P<0.001; one-way ANOVA, n=3). (C) Quantitative analysis of additional immune factors. Exo treatment

significantly impacted levels of factors like CD markers in PHx groups (*P <0.05, ***P<0.001 vs Sham; *P<0.05, ***P<0.001 vs PHx; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3). Data suggest an immunomodulatory effect.

Fig. 5 Macrophage-Derived Factors Mediate Exosome-Induced Hepatoprotection via ZBP1 Modulation. Role of macrophage polarization and ZBP1 in vitro. (A-E) RAW264.7 macrophages treated alone, with Exo, with Exo + ZBP1 OE, ± co-culture with AML12 hepatocytes. (A) Representative flow cytometry (% CD206+, M2 marker). (B) Arg1 mRNA (qPCR, left) and IL-10/TGF-β protein secretion (ELISA, right). (C) Western blot/quantification of Arg1 protein. (D) Quantitative analysis of M2 polarization (% CD206+ or functional index). Exo promoted M2 polarization (increased Arg1, CD206+, IL-10, TGF-β) compared to RAW alone. ZBP1 OE reversed Exo effects. AML co-culture modulated effects. Significance vs RAW group, RAW+Exo group, AML+RAW group, AML+RAW+Exo group shown as described (*P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3). (E-G) Effects on AML12 hepatocytes. (E) Western blot/quantification of Cleaved-caspase3, Cleaved-GSDMD, p-MLKL in AML12 lysates. (F) AML12 proliferation: EdU staining (Scale bar shown)/quantification of EdU+ ratio. (G) Western blot/quantification of Cyclin D1 in AML12. Factors from Exo-Exposed Macrophages (esp. M2-derived) reduced panoptosis and increased proliferation in hepatocytes. ZBP1 OE in macrophages negated these effects. Significance as described for comparisons vs specific raw

groups (*P < 0.05, **P < 0.01, ***P < 0.001; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3).

Fig. 6 Exosome-Conditioned Macrophage Media Protect Hepatocytes via ZBP1 Repression. Direct evidence of macrophage-secreted factors acting on hepatocytes. AML12 hepatocytes treated unconditioned medium (Control), with media from Exo-treated macrophages (Exo-CM), or Exo-CM ± ZBP1 knockdown (KD). (A) Western blot/quantification showing increased ZBP1 in hepatocytes induced by Control CM was suppressed by Exo-CM; ZBP1 KD further reduced it vs Exo-CM (***P < 0.001 vs Control group, ***P < 0.001 vs Exo-CM group; one-way ANOVA, n=3). (B) Western blot/quantification of Cleaved-caspase3, Cleaved-GSDMD, p-MLKL in hepatocytes. Exo-CM reduced panoptosis markers vs Control CM; ZBP1 KD enhanced suppression (***P < 0.001, **P < 0.01, *P < 0.05 vs groups; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3). (C) Hepatocyte proliferation: EdU staining (Scale bar shown)/quantification of EdU+ ratio. Exo-CM and particularly Exo-CM+ZBP1 KD increased proliferation vs Control (***P < 0.001 vs Control, **P < 0.01 vs Exo-CM; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3). (D) ELISA showing TGF-β secretion level (left) likely correlating with hepatocyte functional indices. Factors in Exo-CM promoted increased TGF-β and function; ZBP1 KD increased this further (**P <0.001 vs Control, **P<0.001 vs Exo-CM; one-way ANOVA followed by Tukey's post-hoc test for multiple comparisons, n=3).