# Experimental fibrosis alters matrix-bound vesicles cargo that do not revert after histologic recovery

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## ABSTRACT

**Background:** Although it is now understood that hepatic fibrosis can resolve, potential therapies to enhance fibrosis resolution are lacking. It was recently shown that a major source of signaling molecules in ECM resides within matrix bound nanovesicles (MBV). MBV are nanometer-sized (~50-300 nm), membranous vesicles bound within the ECM collagen network. Changes in MBV cargo may contribute, at least in part, to the phenotypic changes driven by fibrosis and recovery. The purpose of this study was to determine the impact of experimental fibrosis and recovery on liver-derived MBV. **Methods:** Fibrosis was induced by injecting male C57Bl/6J mice with $CCl_4$ (1 ml/kg 2 ×/wk) for 4 wks; animals were sacrificed 1-28 days later. Liver injury and fibrosis was monitored by clinical chemistry, histology and gene expression. MBV were extracted from livers of wild-type (control), pair-fed (PF) and ethanol-fed C57BL/6 mice. The trypsinized MBV proteome cargo was analyzed by 1D-LC-MS/MS analysis using a Proxeon EASY-nLC 1000 UHPLC and nanoelectrospray ionization into an Orbitrap Elite mass spectrometer (Thermo). Quantitative and qualitative feature data were extracted and analyzed using Proteome Discoverer v2.4 using the UniprotKB database. Hierarchical clustering of significantly-changed MBV proteins was performed using StringDB and KEGG classification. **Results:** $CCl_4$ induced robust bridging fibrosis, which rapidly resolved after cessation, reverting to almost normal histology and expression after 28d recovery (Fig 4,5). The amount and type of proteins associated with the MBV tended to decrease in fibrosis, which was not reversed even after fibrosis recovery (Fig 6,7). Multidimensional Scaling (MDS) analysis formed 3 unique clusters between control, fibrosis and recovery (Fig 8). StringDB and KEGG analysis indicated that the proteins lost in fibrosis/recovery were predominantly RNA-binding proteins associated with the ribosome, which may also impact the RNA cargo of the MBV. **Conclusion:** Liver MBV alter their protein cargo in response to experimental fibrosis. Importantly, these changes do not revert to baseline levels even after almost complete histological recovery of fibrosis. These data suggest MBV may be a novel prospect for mechanistic insight into hepatic disease progression and recovery.

## BACKGROUND

Hepatic fibrosis is a canonical example of ECM dyshomeostasis, leading to accumulation of fibrillary ECM, such as collagen. Although hepatic fibrosis is considered almost synonymous with collagen accumulation, the qualitative and quantitative alterations to the hepatic ECM during fibrosis are much more diverse and certainly occur much earlier than fibrotic scarring of the liver. Homeostasis in ECM is mediated by a balance in the production of ECM, as well as in the degradation of existing ECM by matrix metalloproteinases (MMPs). Even in cases where there is a net increase in ECM in liver (e.g. fibrosis) overall turnover is also increased. It is now well understood that hepatic fibrosis is potentially reversible, which has been identified in experimental models,[1] and later in human cohorts.[2, 3] Several key events can drive fibrosis resolution, such as stellate cell deactivation/apoptosis and changes to the inflammatory/wound healing response;[4] however, potential therapies to enhance fibrosis resolution are lacking.

Matrix-bound nanovesicles (MBV; Figure 2) are nanometer-sized (~50-300nm diameter), membranous vesicles bound within the network of ECM, that protect biologically active signaling molecules (miRNA and protein) from degradation and denaturation.[5] Previous studies have shown that MBV, can modulate phenotypic transition of nearby cells, which can redirect fibrotic responses towards tissue remodeling responses. The ability of MBV to survive even harsh tissue decellularization processes suggests evolutionarily-developed fundamental role in tissue and organ organization across species, and a regulatory role in tissue response to injury. Although it is known that hepatic ECM possess MBV, their functional role has not been elucidated. Moreover, the impact of ECM turnover caused by fibrosis and fibrosis resolution has not been explored. The purpose of the current study was to investigate the impact of experimental fibrosis and fibrosis resolution on the protein cargo of hepatic MBV.

## MATERIALS AND METHODS

**Animals and Treatments.** 8 week old, male C57Bl/6J mice from Jackson Laboratory (Bar Harbor, ME) were housed in a pathogen-free barrier facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care, and procedures were approved by the local Institutional Animal Care and Use Committee. Mice were administered $CCl_4$ (1 ml/kg i.p.; diluted 1:4 in olive oil; Sigma-Aldrich, St. Louis, MO) 2×/wk for 4 wks.[8] Animals were sacrificed 1 (“fibrosis”) and 28 (“recovery) days after final injection (Figure 3). Blood was collected from the vena cava just prior to sacrifice by exsanguination and citrated plasma was stored at -80°C for further analysis. Portions of liver tissue were frozen immediately in liquid nitrogen, while others were fixed in 10% neutral buffered formalin or embedded in frozen specimen medium (Tissue-Tek OCT compound, Sakura Finetek, Torrance, CA) for subsequent sectioning and mounting on microscope slides.

**Biochemical analyses and histology.** Plasma levels of aminotransferases (ALT and AST) were determined using standard kits (Thermo Scientific, Middleton, VA). Paraffin-embedded sections of liver were stained with hematoxylin and eosin (H&E) to assess overall hepatic structure, and sirius-red/fast green was used to assess fibrosis. Staining was quantitated by image analysis as described previously.[10]

**MBV isolation and LC-MS/MS proteome analysis.** MBV were isolated from liver using an established digestion and ultra-centrifugation protocol and further counted by nano-tracking analysis.[7] MBV pellets were resuspended in 4% SDS supplemented with 1X HALTTM (ThermoFisher) protease/phosphatase inhibitor cocktail prior to tryptic digestion using the S-trapTM (Protifi, Farmingdale, NY) procedure. Digested peptide was recovered, dried by speedvac and resuspended in 50uL distilled deionized water. Peptides (200ng) were analyzed by 1D-LC-MS/MS analysis using a Proxeon EASY-nLC 1000 UHPLC and nanoelectrospray ionization into a Q-Exactive HF mass spectrometer (Thermo) with FTMS1 data collected at 200,000-240,000 resolution. Qualitative (de novo spectral assignment of amino acid sequences) and quantitative (AUC from high resolution extracted ion chromatograms) peptide features data were developed from MSn datasets using PEAKS Studio X (v10).

**Statistics.** Quantitative data are reported as either means ± SEM (n=5–6). Analysis of variance followed by Tukey’s multiple-comparison test were used to evaluate differences between the experimental groups. Statistical analysis was performed using GraphPad Prism version 8.2.0 for Windows (GraphPad Software, Inc., La Jolla, CA). A P-value of 0.05 was selected before the study as the level of significance.

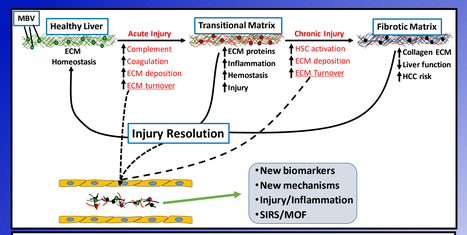


**Figure 1: Transitional Remodeling of Hepatic ECM.** Acute injury causes formation of transitional ECM, which may contribute to injury and inflammation. Transitional ECM often resolves after removal of the insult and may contribute to recovery from that insult. With continued injury, transitional matrix may progress to fibrotic matrix, which can also resolve. Even though ECM accumulates after liver injury, net turnover of the ECM is also increased. MBV are key mediators of ECM-driven phenotype. We show here that MBV protein cargo is altered by either experimental fibrosis and the overarching goals of this project are to build on these data to explore MBV role in ECM remodeling in fibrosis.

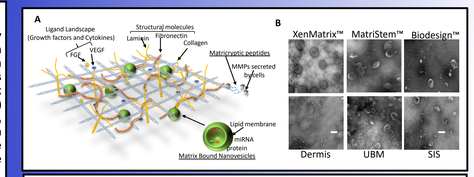


**Figure 2: MBV isolated from ECM Bioscaffolds.** (A) Graphic representation of MBV embedded within the ECM. (B) Transmission electron micrographs of MBV isolated from laboratory produced porcine urinary bladder matrix (UBM), small intestinal submucosa (SIS), and dermis; and three commercially available ECM bioscaffold equivalents: ACell® MatriStem™ (porcine UBM), BD® XenMatrix™ (porcine dermis), and Cook Biotech® Biodesign™ (porcine SIS).

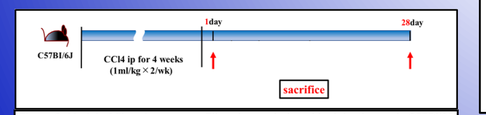


**Figure 3: Model of fibrosis recovery.** Fibrosis was induced by injecting male C57Bl/6J mice with $CCl_4$ (1 ml/kg 2×/wk) for 4 wks; animals were sacrificed 1, 28 days later.

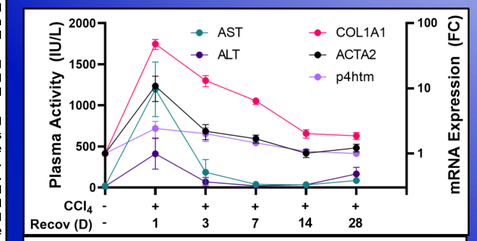


**Figure 4: Recovery from liver injury and fibrosis.** Liver transaminase levels (AST and ALT) were determined in plasma. mRNA expression of fibrosis genes (COL1A1, ACTA2 and p4htm) were determined by qPCR.

Routine indices of liver injury and hepatic fibrosis rapidly resolved after cessation of $CCl_4$ intoxication, returning to almost normal levels after 28 d of recovery.

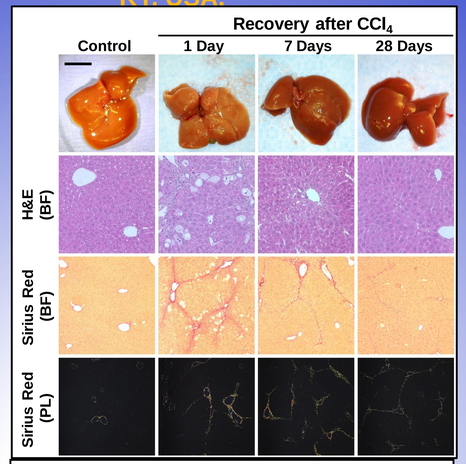


**Figure 5: Recovery from fibrosis.** Mice were exposed to $CCl_4$ for 4 weeks and sacrificed 1-28 days after exposure. Paraffin embedded liver sections were stained with H&E and sirius red (brightfield and polarized light).

4 wks of $CCl_4$ induced severe liver injury and bridging fibrosis. Recovery led to a progressive loss of these changes. Although recovery was almost complete, thin septa of fibrosis were still present 28 d after cessation of injury, despite normal routine indices of fibrosis and liver injury (see Figure 4).

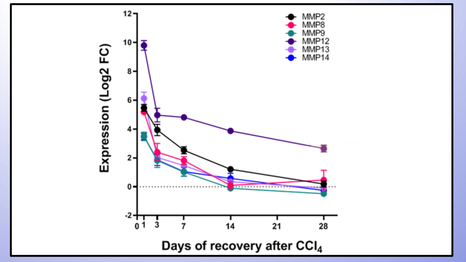


**Figure 6: Hepatic expression of MMPs.** Mice were treated and qPCR for mRNA expression of MMPs were performed. Data are reported as Log2FC.

Even under cases where ECM accumulates, such as hepatic fibrosis, there is a net increase in the turnover of the ECM, as evidenced by induction of ECM-metabolism enzymes, such as the MMPs. This turnover may impact the protein cargo of the MBVs.

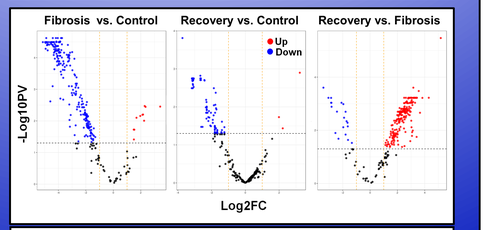


**Figure 7: Proteomic analysis of MBV in fibrosis and fibrosis recovery mice.** Volcano plots showing downregulation of MBV proteins in fibrosis and recovery compared with controls, and upregulation of proteins in recovery vs fibrosis.

Hepatic fibrosis caused a dramatic change in the protein cargo of the MBV, with most proteins quantitatively decreased (left). Recovery tended to increase MBV protein cargo (compared to fibrosis; right), but was decreased compared to control (middle). These data indicate that MBV protein cargo changes in response to hepatic injury.

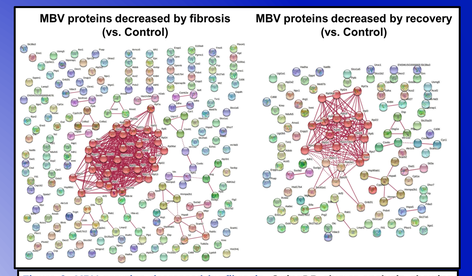


**Figure 8: MBV proteins decreased by fibrosis.** StringDB cluster analysis showing decreased proteins in liver MBV during fibrosis (left) and fibrosis recovery (right) compared to controls. The red nodes and cluster represent ribosomal proteins.

The net loss of protein cargo in the MBVs was driven predominantly by proteins associated with ribosomes, which are known to be key for mediating quantitative and qualitative changes in the RNA cargo of EVs. The main class of proteins lost in MBVs during recovery still clustered as ribosomal proteins.

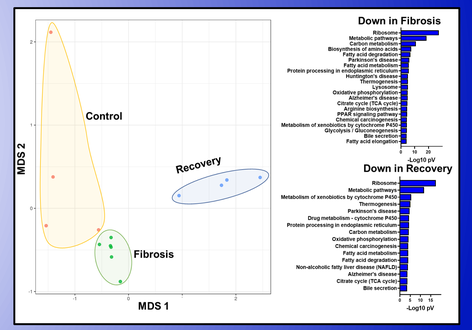


**Figure 9: Proteomic analysis of MBV in fibrosis and fibrosis recovery mice.** Multidimensional Scaling (MDS; left) plot shows separation of MBV proteome between the groups. KEGG analysis (right) of significantly changed proteins in MBVs from fibrosis (upper) and recovery (lower) are shown.

Fibrosis dramatically changed the MBV protein content. Although recovery from fibrosis was nearly complete (see Figure 5), the protein cargo in the MBVs did not revert to control. Analogous to StringDB analysis, the key pathway impacted (by KEGG analysis) was in proteins associated with ribosomal metabolism and processing in both fibrotic and recovery MBVs.

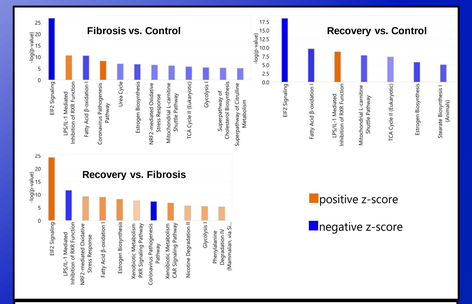


**Figure 10: Pathway enrichment of proteome in MBVs.** Ingenuity Pathway Analysis (IPA) of canonical pathways of significantly changed proteins between groups are shown. -Log10PV>5. Absolute Z-score >1.

IPA identified that fibrosis caused a robust loss of proteins involved in EIF2 signaling, a stress-related master controller of RNA processing. IPA also identified a strong increase in proteins involved in LPS/IL-1 signaling. Although recovery reversed several of these changes, the reversion was incomplete.

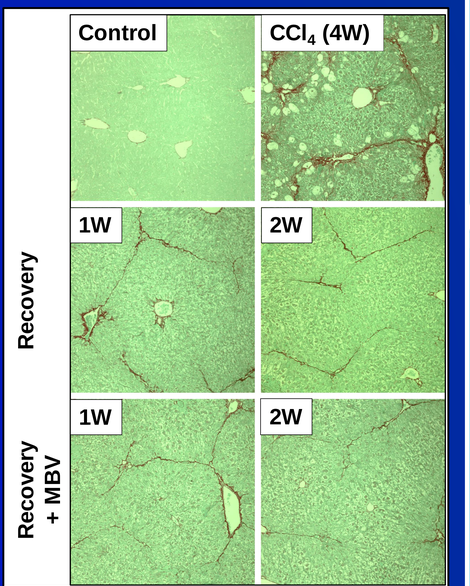


**Figure 11: Exogenous “healthy” MBV accelerated recovery from fibrosis.** Mice were injected with CCl4 for 4 weeks and then allowed to recover 1-2 weeks. The effect of exogenous “healthy” MBV (30 mg/mouse i.p. 2x/week) from porcine urinary bladder was compared to vehicle (saline) alone. Representative photomicrographs (10x) of livers stained with Sirius Red/fast green are shown.

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## SUMMARY

- Hepatic fibrosis and liver injury recovered after 28 d.
- MBV-associated proteins decreased in fibrosis.
- Changes of the amount and type of MBV-associated proteins was not completely reversed even after fibrosis recovery.
- The proteins lost in fibrosis/recovery were predominantly RNA-binding proteins associated with the ribosome.
- Exogenous “healthy” MBV accelerated recovery from fibrosis.

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