Therapeutic effects of serum extracellular vesicles in liver fibrosis

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ABSTRACT
The lack of approved therapies for hepatic fibrosis seriously limits medical management of patients with chronic liver disease. Since extracellular vesicles (EVs) function as conduits for intercellular molecular transfer, we investigated if EVs from healthy individuals have anti-fibrotic properties. Hepatic fibrogenesis or fibrosis in carbon tetrachloride (CCl4)- or thioacetic acid-induced liver injury models in male or female mice were suppressed by serum EVs from normal mice (EVN) but not from fibrotic mouse (EVF). CCl4-treated mice undergoing EVN therapy also exhibited reduced levels of hepaticocyte death, inflammatory infiltration, circulating AST/ALT levels and hepatic or circulating pro-inflammatory cytokines. Hepatic histology, liver function tests or circulating proinflammatory cytokine levels were unaltered in control mice receiving EVN. As determined using PKH26-labelled EVN, principal target cells included hepatic stellate cells (HSC; a normally quiescent fibroblastic cell that undergoes injury-induced activation and produces fibrosis during chronic injury) or hepatocytes which showed increased EVN binding after, respectively, activation or exposure to CCl4. In vitro, EVN decreased proliferation and fibrosis-associated molecule expression in activated HSC, while reversing the inhibitory effects of CCl4 or ethanol on hepatocyte proliferation. In mice, microRNA-34c, -151-3p, -483-5p, -532-5p and -687 were more highly expressed in EVN than EVF and mimics of these microRNAs (miRs) individually suppressed fibrogenic gene expression in activated HSC. A role for these miRs in contributing to EVN actions was shown by the ability of their corresponding antagonists to individually and/or collectively block the therapeutic effects of EVN on activated HSC or injured hepatocytes. Similarly, the activated phenotype of human LX-2 HSC was attenuated by serum EVs from healthy human subjects and contained higher miR-34c, -151-3p, -483-5p or -532-5p than EVs from hepatic fibrosis patients. In conclusion, serum EVs from normal healthy individuals are inherently anti-fibrogenic and anti-fibrotic, and contain microRNAs that have therapeutic actions in activated HSC or injured hepatocytes.

Abbreviations: ALT: alanine aminotransferase; AST: aspartate aminotransferase; CCl4: carbon tetrachloride; CCN2: connective tissue growth factor; E: eosin; EGFP: enhanced green fluorescence protein; EVs: extracellular vesicles; EVF: serum EVs from mice with experimental hepatic fibrosis; EVN: serum EVs from normal mice; H: hematoxylin; HSC: hepatic stellate cell; IHC: immunohistochemistry; IL: interleukin; MCP-1: monocyte chemotactic protein-1; miR: microRNA; mRNA: messenger RNA; NTA: nanoparticle tracking analysis; PCNA: proliferating cell nuclear antigen; qRT-PCR: quantitative real-time polymerase chain reaction; SDS-PAGE: sodium dodecyl sulphate – polyacrylamide gel electrophoresis; aSMA: alpha smooth muscle actin; TAA: thioacetinic acid; TG: transgenic; TGF-β: transforming growth factor beta; TEM: transmission electron microscopy; TNFa: tumour necrosis factor alpha.

Introduction
Chronic liver diseases such as hepatitis, alcoholic liver disease and non-alcoholic fatty liver disease are the cause of considerable morbidity and mortality and affect millions of individuals worldwide [1]. The long-term nature of the inflammatory and injury processes in these diseases is frequently associated with the development of hepatic fibrosis, a pathology in which insoluble collagen and matrix components are deposited in excessive amounts in the interstitial spaces, which can severely compromise liver function [2]. While fibrosis may reduce or resolve in some individuals after treatment of the underlying disease, many patients are non-responsive to this approach and are reliant on direct fibrosis therapy for which approved

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drugs are lacking [3,4]. A new lead in liver fibrosis research has come from studies of extracellular vesicles (EVs) which are derived from numerous cell types in the body and comprise exosomes, microvesicles and apoptotic bodies [5]. Exosomes and microvesicles are able to mediate cell–cell transfer of their respective molecular payloads (microRNA [miR], messenger RNA [mRNA], proteins) resulting in transcriptional or translational modifications in the recipient cells [5,6]. EVs may either drive or dampen pathogenetic pathways in various liver cells depending on the phenotype of the donor and recipient cells and the nature of the molecular information transferred between them [7].

After their cellular release, EVs enter the intercellular space and may be taken up by neighbouring cells or they may enter body fluids such as blood, urine or saliva and can potentially be delivered to target cells that reside at appreciable distances from their sites of release [5]. EVs in plasma or serum are highly heterogeneous and originate from many circulating cell types (e.g. erythrocytes, leucocytes, platelets, megakaryocytes, monocytes, granulocytes, lymphocytes) as well as from cells in tissues and organs that have direct (e.g. endothelial cells) or indirect contact (via interstitial fluids) with the systemic circulation [8]. Serum or plasma EVs are a readily accessible and rich source of biomarkers that have potential for assessing organ disease, including that of the liver [9-14], because the pathogenic changes involved may be reflected in an altered EV molecular payload that can be detected by comparison to EVs from healthy individuals or from individuals at a different disease stage. However, little attention has been paid to the functional properties of EVs from the body fluids of individuals in good health. In this study, we show that serum EVs from healthy individuals are therapeutic for liver fibrosis due to their ability to attenuate activation of hepatic stellate cells (HSC, the principal fibrosis-producing cell in the liver), hepatocyte injury and inflammation. The direct therapeutic properties of serum EVs on HSC or hepatocytes appear to be due, at least in part, to the action of EV miRs that are suppressed in serum EVs during fibrosis.

Materials and methods

Collection of human serum

Human blood was collected from healthy volunteers (9 males, 9 females; age range 23–28 years) using a protocol approved by the Institutional Review Board of Nationwide Children’s Hospital (Columbus, OH). Exclusion criteria for blood donation included the presence of known acute or chronic diseases, the taking of medications, the consumption of alcohol within the preceding 24 h, or age less than 21 years or greater than 30 years. Blood samples were de-identified prior to being received for processing. Blood was collected into SST™ tubes (BD Vacutainer®, ThermoFisher, Waltham, MA) from which serum was obtained by low speed centrifugation and stored for up to 1 month at -80°C prior to EV purification. Serum samples from hepatitis B virus patients with F3/4 fibrosis ($n = 12$) or age- and sex-matched healthy control subjects ($n = 12$) were obtained from The Liver Research Center, Beijing Friendship Hospital, Capital Medical University (Beijing, China). Samples were collected under an IRB protocol approved by the Ethical Committee of Beijing Friendship Hospital, Capital Medical University in support of clinical trial NCT01938781.

Collection of mouse serum

All animal protocols were approved by the Institutional Animal Care and Use Committee of Nationwide Children’s Hospital (Columbus, OH). Blood was collected by cardiac puncture or retro-orbital bleed in control male or female wild-type Swiss Webster or FVB mice (6–8 weeks) or in mice of the same strain in which liver fibrosis was induced, with or without EV therapy, as described below. Blood was allowed to clot and serum was collected in SST™ tubes (ThermoFisher), pooled within each strain or treatment group, and used for assay or EV isolation immediately or after storage at -80°C for up to 6 months [15].

Serum EV purification

Human or mouse sera underwent low-speed centrifugation (300 × g, 15 min; 10,000 × g, 30 min, 4°C,) and the supernatants were passed through a 0.22 μm filter (Merck, Darmstadt, Germany) and centrifuged (10,000 × g, 30 min 4°C) to remove particulates. The supernatant was then subjected to ultracentrifugation (100,000 × g for 90 mins at 4°C) in a T-70i fixed-angle rotor (Beckman Coulter, Brea, CA) to pellet the small vesicles [16–18]. The pellet was then rinsed with PBS to remove contaminating proteins and ultracentrifuged using the same conditions after which the supernatant was discarded and the EV-containing pellet was recovered. For some experiments, mouse serum EVs were isolated using PureExo® kits (101Bio, Palo Alto, CA) [19]. EVs from both purification procedures were resuspended in PBS. EVs purified from the serum of normal or fibrotic mice are hereafter termed “E VN”...
or “EVF”, respectively. For the therapeutic studies described, serum typically was pooled from 10–20 mice from which EVs were then isolated. Over the course of these experiments, 15 such pools were collected with consistent therapeutic outcomes irrespective of the actual pool used, with each pool being tested in at least triplicate among the various assays. For human EV characterization and biological assay, serum EVs from the 18 donors were individually purified and analysed after which some of the data were then combined for analysis.

**EV characterization**

Purified EVs were diluted to $10^6$–$10^7$ particles/ml in PBS for nanoparticle tracking analysis (NTA) using a Nanosight 300 equipped with v3.2.16 analytical software (Malvern Instruments, Westborough, MA). Two videos (25 s each) were recorded for each sample and the software was used to estimate concentration and size of the particles. The recordings were performed at room temperature which was monitored manually. Camera gain was 15 and the shutter speed was 4.13 ms. For analysis, the detection threshold was set to 6. Calibration was carried out using 100 nm polystyrene latex microspheres (Magsphere Inc., Pasadena, CA) diluted to a known concentration in PBS and then two videos were recorded.

For analysis by transmission electron microscopy (TEM), 200-mesh, copper grids coated with a Formvar/Carbon support film (Ted Pella, Redding CA) were glow-discharged with a Pelco easiGlow discharge cleaning system (Ted Pella) prior to use. EV samples were fixed in 2.5% glutaraldehyde for 30 min and pelleted by ultracentrifugation and then placed on the grids which were subsequently negative-stained using 1% aqueous uranyl acetate. Grids were examined using a FEI Tecnai G2 Biotwin TEM (Atlanta, GA) operating at 80 kV and digital micrographs were captured on an AMT side-mount camera with FEI imaging software.

EV proteins (10–25 μg) were analysed by sodium dodecyl polyacrylamide gel electrophoresis (SDS-PAGE) or Western blot. Gels were stained with Coomassie Blue or subjected to Western blot using primary antibodies to CD81 (1:400; ProSci, Poway, CA), CD9 (1:300; Lifespan Bioscience Inc., Seattle WA), CD63 (1:400; Abcam, Cambridge, UK), flotillin-1 (1:500; Abcam) or asialoglycoprotein receptor 1 (ASGPR1) (1:500, GeneTex, Irvine, CA) [16,17,19,20]. For organ localization and cell binding studies, EVN were labelled with PKH26 lipophilic membrane red fluorescent dye (Sigma-Aldrich) as described [17].

**CCL₄-induced hepatic fibrosis in mice**

Male or female Swiss Webster wild-type or transgenic (TG) Swiss Webster mice (Stock TG (connective tissue growth factor-enhanced green fluorescent protein [CCN2-EGFP]) FX156GSat/Mmucd expressing EGFP under the control of the CCN2 promoter [21]) (TG CCN2-EGFP) mice (4–5 weeks; $n = 5$ per group) received i.p. carbon tetrachloride (CCl₄; 175 μl in 1325 μl corn oil/kg; Sigma-Aldrich, St Louis, MO) on Days 1, 3, 5, 7 and 9. Control mice received i.p. corn oil (1500 μl/kg) alone on the same days. Some mice received i.p. EVN (0–40 μg EVN protein per g body weight) on Days 2, 4, 6 and 8. Mice were sacrificed on Day 10 and liver lobes were either perfused with PBS, fixed in 4% paraformaldehyde and processed for histological analysis or immediately harvested for EGFP imaging using a Xenogen IVIS 200 (PerkinElmer, Waltham, MA).

**CCL₄- or TAA-induced hepatic fibrosis in mice**

Wild-type male Swiss Webster mice or FVB (4–5 weeks; $n = 5$ per group) received i.p. CCl₄ (175 μl in 1325 μl corn oil/kg) or corn oil (1500 μl/kg) three times per week for 5–6 weeks. During the last 2–3 weeks, some mice received i.p. EVN (0–40 μg/g) every other day 3 times per week, on alternative days to those used for CCl₄ or oil administration. Mice were sacrificed 36 h after the last CCl₄ or oil injection, or in non-treated littermates. Just prior to sacrifice in some animals, blood was collected by cardiac puncture and serum obtained for performing liver function tests, assessing cytokine levels or EV purification. Individual liver lobes were harvested and snap-frozen in liquid nitrogen for subsequent RNA extraction or perfused using PBS followed by 4% paraformaldehyde (Sigma-Aldrich, St Louis, MO) for histological analysis of fixed tissue. In some experiments, CCl₄- or oil-treated FVB mice received a single i.v. injection of PKH26-labelled EVN (40 μg/g) 4 h prior to sacrifice after which PKH26 fluorescence was detected in (i) freshly isolated liver pieces by direct Xenogen imaging; (ii) 6 μm sections of frozen liver tissue by fluorescence microscopy (Axiovert 25, Zeiss, Obercochen, Germany) or (iii) in 24-h cultures of primary HSC or hepatocytes by confocal microscopy (Model 710, Zeiss) using our published procedures [16]. As an alternative method of inducing liver fibrosis, wild-type male FVB mice (4–5 weeks; $n = 10$ per group) received i.p. thioacetic acid (TAA; 100 mg/kg; Sigma-Aldrich) in saline 3 times per week for 6 weeks. Control mice received
i.p. saline alone. Over the last week, some mice received i.p. EVN (40 μg/g) every day for 6 days and were sacrificed 60 h later. Livers were processed as described above.

**HSC or hepatocyte cultures**

Livers from control or CCl₄-treated male wild-type Swiss Webster mice (6–8 weeks) were used for isolation of primary HSC or hepatocytes. Livers were perfused in situ and then subjected to either collagenase digestion for isolation of hepatocytes [16] or pronase/collagenase digestion and buoyant-density centrifugation for isolation of HSC [16,22,23]. As we have previously described [19], quiescent HSC isolated from control animals contained lipid droplets and were positive for desmin or Twist1, but not for CCN2, αSMA or collagen α(I) whereas activated HSC isolated from animals treated with CCl₄ were positive for desmin, CCN2, αSMA or collagen α(I), but not for Twist1 (data not shown). Hepatocytes were maintained in complete William E medium (Gibco, Billings, MT) and used within the first 3 days of primary culture while HSC were split 1:3 in DMEM/F12/10% FBS medium (Gibco) every 5 days and used at up to passage 4 (P4). LX-2 human HSC (a kind gift from Dr Scott Friedman, Icahn School of Medicine at Mount Sinai, New York, NY) were cultured in DMEM/10% FBS as described [19,22]. AML12 mouse hepatocytes (American Type Culture Collection, Manassas, VA) were cultured in DMEM/F12/10% FBS supplemented with insulin, transferrin, selenium and dexamethasone [19].

**Histology**

Perfused mouse livers were fixed and embedded in paraffin. Sections of 5 μm thickness were cut and stained with H and E. Collagen was detected by staining sections with 0.1% Sirius Red (Sigma-Aldrich). Immunohistochemistry (IHC)

Mouse liver sections were incubated with primary antibodies to albumin (1:400, Bethyl Laboratories, Inc., Montgomery, TX), Ki67 (1:500, Thermo Fisher, Grand Island, NY), F4/80 (1:100, Abcam, Cambridge, MA), Ly6c (1:400, Abcam), CD45 (1:150, Abcam), monocyte chemotactic protein-1 (1:100, Abcam), TNFα (1:150, Abcam), IL1-β (1:100, Abcam), collagen α1 (1:250, Abcam), αSMA (1:1000; Dako Cytomation, Glostrup, Denmark) or CCN2 (in-house; 5 μg/ml [21,24]) followed by Alexa Fluor 488 goat-anti rabbit IgG and Alexa Fluor 568 goat-anti mouse IgG, or Alexa Fluor 647 goat-anti mouse IgG, or Alexa Fluor 568 goat-anti-chicken IgG (all at 1:1000; Thermo Fisher) for 1 h at room temperature. The slides were mounted with Vectashield Mounting Medium containing 4’,6-diamidino-2-phenylindole nuclear stain (Vector Laboratories, Burlingame, CA) and examined by confocal microscopy. Activated HSC were identified by positive immunostaining for CCN2, αSMA and/or collagen α(I).

**Alanine aminotransferase (ALT) or aspartate aminotransferase (AST) assay**

Mouse serum or hepatocyte conditioned medium were analysed using ALT or AST assay kits (Sigma) according to the manufacturer’s instructions.

**Multiplex cytokine assay**

Cytokine levels in mouse serum were determined using a Mouse Proinflammatory Panel 1 Kit (Meso Scale Discovery, Rockville, MD) according to the manufacturer’s instructions. All components of the kit were brought to room temperature and the antibody-coated plate was shaken with diluent for 30 min at 800 rpm. Two-fold dilutions of the serum samples were loaded in duplicate on to the plate alongside serial dilutions of the calibrator used to construct standard curves for each analyte. Plates were incubated with shaking for 2 h after which the wells were washed and incubated with labelled detection antibodies for 2 h. After washing, read buffer was added to develop the plate which was then read in a Sector Imager 2400 using Discovery Workbench Software (Meso Scale Discovery).

**Mouse cytokine microarray**

A mouse cytokine proteome profiler™ array (Panel A kit; R & D Systems, Minneapolis, MN) was used to detect 40 different cytokines or chemokines in liver extracts according to the manufacturers’ instructions. Each array was incubated with 267 μg/ml pooled liver tissue lysates from control or fibrotic Swiss Webster mice (5-week CCl₄ model) that had or had not received EVN therapy as described above (n = 5 mice per group). Arrays were developed using chemiluminescence and the mean signal (pixel density) for each pair of duplicate component spots was quantified by scanning.
**EV binding assays**

PKH26-labelled EVN were added for 16 h to Day 1 or P3 mouse HSC or to Day 2 primary mouse hepatocytes with or without 40-h exposure to CCl₄ (20 mM in 0.1% DMSO) or DMSO carrier (0.1%). PKH26-labelled human serum EVs were added for up to 24 h to LX-2 cells. Cells were then washed in PBS and lysed in lysis buffer (ThermoFisher). Cell-associated PKH26 fluorescence was measured at 590/540 nm using a Spectra Max® M2 microplate reader (VWR, Atlanta, GA, USA) and normalized to total cellular protein determined by bicinchoninic acid assay (ThermoFisher).

**Effect of serum EVs on HSC**

P2-3 mouse HSC were seeded in 6-well plates for 24 h in DMEM/10% FBS, cultured in serum-free medium for 12–24 h and treated with EVN or EVF (0–8 μg/ml) for up to 24 h. LX-2 cells were grown for 24 h in DMEM/10% FBS and then cultured in serum-free medium for the next 24 h. Some LX-2 cells were treated with 0–10 ng/ml TGF-β for 36 h, the last 24 h of which included co-incubation with human serum EV. Expression of CCN2, αSMA or collagen α1(I) mRNA was determined by qRT-PCR; the presence of αSMA was determined by IHC and cell proliferation was determined using a CyQUANT® assay (Thermo Fisher).

**Effect of serum EVs on hepatocytes**

AML12 hepatocytes were incubated for 24 h in DMEM/F12/10% FBS medium (plus supplements) for 24 h, serum-starved for 12 h, treated with 0–20 mM CCl₄ for 48 h in the presence or absence of EVN (8 μg/ml) for the last 24 h and assessed for cell proliferation using a CyQUANT® assay. Culture medium was assessed for ALT and AST as described above.

**RNA extraction and RT-qPCR**

Total RNA from liver tissues or cultured HSC was extracted using a microRNeasy Plus kit (Qiagen, Valencia, CA) and reverse transcribed using a miScript II RT kit (Qiagen) according to the manufacturers’ protocols. Resulting transcripts were analysed by qRT-PCR as described [17] with primers for EGFP, CCN2, αSMA, collagen α1(I) or SMAD3 (Table 1). Each reaction was run in triplicate, and all samples were normalized to glyceraldehyde-3-phosphate dehydrogenase. Negative controls were a non-reverse transcriptase reaction and a non-sample reaction.

**Differential EV miR analysis**

Using a ABI-7900 qPCR machine (Applied Biosystems, Forster City, CA) miR profiling was performed on PureExo®-purified EVs obtained from pooled serum from Swiss Webster mice (200 μl serum per mouse; 5 mice per group (control, 5-week oil, 5-week CCl₄) using a mouse miRnome Array kit (Qiagen). Differential comparison of the miRs that were up- or down-regulated in each group was performed using miR array software (Qiagen) to identify those miRs that were expressed at higher levels in EVN versus EVF, that underwent the greatest suppression of expression in EVF as compared to EVN, but that were unchanged between EVN and oil-treated mice. Data were confirmed by RT-PCR using primer sequences shown in Table 1. The same strategy, using a human miRnome Array (Qiagen), was used to establish differential serum EV miR expression between liver fibrosis patients and healthy control subjects. Serum (0.5 ml) from each patient (control, fibrosis) was individually processed to purify EVs from which small RNA was then isolated and reverse-transcribed. Samples in each group were then pooled for miR profiling.

### Table 1. Primers used for RT-PCR.

| Gene (accession number) | Gene (GenBank accession number) | Sense primers | Anti-sense primers | Product size (bp) |
|-------------------------|---------------------------------|---------------|---------------------|------------------|
| CTGF (mouse) (NM 010217) | 5’ CATTCTGCAGTGGACTGTT3’ | 5’ AAGATGTGATGGCCACAGG 3’ | 111 |
| Collagen α1(I) (mouse) (NM 007742) | 5’ GCCGAACCTCCAAAGGAGGC3’ | 5’ CTGGGAGGCTTGGTGACATTAG 3’ | 148 |
| αSMA (mouse) (NM 007392) | 5’ GCTCTGAGCTCTGAAAGG 3’ | 5’ CTCGTTCCTGCTGGTACAT 3’ | 148 |
| Smad3 (mouse) (AF016189.1) | 5’ CTGGGGCCTACTGCTTAATG 3’ | 5’ GACACAAATTCCTGGTTGT3’ | 239 |
| GAPDH (mouse, human) (NM 002046) | 5’ TGGACACCAACTCTT3’ | 5’ GCACAGATGGTGAGTCTCC 3’ | 87 |
| Collagen α1(I) (human) (NM 0000088) | 5’ GAAGCCGTGTCTCCCTT3’ | 5’ GAAGCAGTGGTCTCGAGA 3’ | 91 |
| EGFP (MIMAT00004580) | 5’ GGAGGACGGCGAACTACACAA 3’ | 5’ AAGTCGATGCCCTTCAGC 3’ | 101 |
| miR34c-3p (MIMAT00004580) | Universal anti-sense | | 22 |
| miR151-3p (MIMAT000061) | Universal anti-sense | | 21 |
| miR483-5p (MIMAT0004782) | Universal anti-sense | | 22 |
| miR523-5p (MIMAT0002889) | Universal anti-sense | | 21 |
| miR6287 (MIMAT0003466) | Universal anti-sense | | 22 |
Transfection of HSC or hepatocytes with miR mimics or antagonors

P2-P3 mouse HSC or AML12 hepatocytes were transfected individually or collectively with 100 nM miR mimics (miRs-34c-3p, -151-3p, -483-5p, -532-5p, -687) or their corresponding antagonors (Qiagen) by electroporation (Nucleofector Kit, Lonza, Koln, Germany) as described [16]. Each antagonor preparation comprised chemically synthesized single-stranded modified RNAs that specifically inhibit the function of the mature miR target. Control transfections were performed using a commercial scrambled miR mimic or antagonor (Qiagen). MiR-transfected HSC were incubated for 24 h in DMEM/F12/10% FBS medium and then analysed by qRT-PCR. Antagonor-transfected HSC were incubated for 24 h in DMEM/F12/10% FBS medium, serum-starved for 12 h, treated with EVN (8 μg/ml) for 24 h and then analysed by RTPCR or CyQUANT® cell proliferation. Antagonor-transfected AML12 hepatocytes were treated with 0–20 mM CCl₄ for 48 h in the presence or absence of EVN (8 μg/ml) and assessed for cell proliferation as described above.

Statistical analysis

All experiments were performed at least three times with triplicate measurements, with data expressed as mean ± S.E.M. Fluorescence images were scanned and quantified using Image J software (NIH, Bethesda, MD). The data from qRT-PCR, NTA, imaging, multiplex cytokine analysis and ALT/AST assays were analysed by Student’s t-test using Sigma plot 11.0 software (SPSS Inc., Chicago, IL). P values < 0.05 were considered statistically significant.

Results

Characterization of serum EVs from normal or CCl₄-treated mice

Vesicles isolated from the serum of normal wild-type Swiss Webster mouse (“EVN”) using sequential ultracentrifugation were 115 ± 8 nm in diameter and positive for CD81, CD9 and flotillin-1 (Figure 1(a)). As assessed by TEM, the isolated EVs were approximately 100 nm, had a spherical morphology and contained electron-dense material within a limiting membrane (Figure 1(a)). Very similar characteristics were also evident when the serum of wild-type Swiss Webster mice was alternatively processed using ExoPure® isolation kits (Figure 1(b)). Similar features were again observed for EVs isolated from the serum of TG CCN2-EGFP Swiss Webster mice or wild-type FVB mice (Figure 1(c, d)). Serum EVs from wild type Swiss Webster mice that had been exposed to CCl₄ for up to 5 weeks were also positive for CD81, CD9 or flotillin-1 but appeared to have membrane irregularities as assessed by TEM (Figure 1(e)) and underwent a progressive decrease in their concentration and size during CCl₄ treatment (Figure 1(e, f)). Serum EVs collected from fibrotic mice after 5 weeks of CCl₄ treatment (“EVF”) were approximately 10% fewer in number and 20% smaller than EVN from control animals (Figure 1(e, f)). These changes were not evident for serum EVs isolated from control animals treated with oil (Figure 1(f)). Although the predominant EV proteins did not appear to change qualitatively or quantitatively between EVN and EVF as assessed by SDS-PAGE analysis, Western blot analysis showed that the levels of ASPGR1 or CD81 were diminished in EVF to, respectively, 40% or 60% of their levels in EVN (Figure 1(g)).

EVN reduce fibrogenesis and fibrosis in experimental models in vivo

Swiss Webster TG CCN2-EGFP mice were treated on Days 1,3,5,7 and 9 with corn oil or CCl₄ and received EVN or EVF i.p. on Days 4, 6 and 8, with EV dose normalized to EV protein concentration. As assessed on Day 10, expression of EGFP (a surrogate marker for CCN2 – a profibrogenic molecule that is synthesized downstream of transforming growth factor beta (TGFβ) and which is produced principally in activated HSC during liver injury [25]) was dose-dependently inhibited in CCl₄-injured liver by EVN as assessed by direct imaging of freshly harvested liver from female mice (Figure 2(a)) or by IHC for EGFP in male mice (Figure 2(b)), with EV doses of 40 μg/g reducing the fluorescence to levels that were not significantly different from control oil-treated animals. In contrast, CCl₄-induced hepatic EGFP expression was not inhibited by the same dose (40 μg/g) of EVF (Figure 2(b)). Moreover, immunostaining for CCl₄-induced α-smooth muscle actin (αSMA) in activated HSC was reduced in male mice receiving EVN but not EVF (Figure 2(c)). Since male and female mice responded to treatment with EVN and since EVF was not therapeutic, subsequent mouse studies were undertaken in male recipients treated with EVN from male donors.

Experimental fibrosis was established in mice by chronic exposure (5 weeks) to CCl₄ with EVN administered every other day for the last 2 weeks. In TG CCN2-EGFP mice, the CCl₄-induced increase in hepatic EGFP was substantially reduced by EVN (Figure 3(a)) as was the severity and extent of hepatic fibrosis as assessed by Sirius red staining for collagen (Figure 3(b)). Hematoxylin and
eosin (H and E) staining showed that CCl₄-treated mice demonstrated significant levels of hepatocyte disorganization and cellular infiltration whereas these features were not evident in CCl₄-treated mice that received EVN (Figure 3(c)). EVN administration also resulted in suppressed hepatic mRNA expression of collagen α₁(I), αSMA, or CCN2 in CCl₄-treated TG CCN2-EGFP mice (data not shown) or their wild-type counterparts (Figure 4(a)). Furthermore, the high level of caspase 3 and αSMA in activated HSC in fibrotic mice was diminished to control levels in EVN treated mice (Figure 4(b)). In CCl₄-treated mice, EVN treatment also resulted in increased hepatocyte
proliferation and decreased liver injury as shown by, respectively, enhanced staining for Ki67 in albumin-positive cells (Figure 4(b)) and decreased circulating ALT or AST levels (Figure 4(c)). Moreover, EVN treatment caused restitution of serum EV number to pre-CCl4 treatment levels (Figure 4(d)). In control animals, EVN had no effect on hepatic histology, the extent or pattern of Sirius red staining, expression of fibrosis-, apoptosis- or proliferation-associated molecule expression, or circulating levels of AST or ALT (Figure 3(b, c), Figure 4(a–d)). Finally, in TAA-induced liver fibrosis in FVB mice, EVN attenuated hepatic collagen α1(I), αSMA or CCN2 mRNA levels as assessed by qRT-PCR (Figure 4(e)) and reduced tissue damage and inflammation or collagen α1 production as assessed, respectively, by H and E staining or IHC (Figure 4(f)).
Collectively, these data showed that reconstitution of three strains of male or female mice with EVN resulted in a reversal of fibrosis in two different experimental models. This response was associated with decreased expression of fibrogenic, activation or apoptotic genes in HSC, decreased liver injury, increased expression of proliferation markers in hepatocytes and restoration of serum EVs to pre-injury concentrations. Normal control animals exhibited no alterations in hepatic histology, inflammation or liver function following EVN administration.

**EVN dampen CCL₄-induced inflammation**

Exposure of mice to CCl₄ for 6 weeks caused a significant increase in the circulating levels of interferon-γ, TNFα, IL-10, IL-12p70, IL-2, IL-5, IL-4 or IL-6, all of which were reduced to baseline levels by treatment over the last 3 weeks with EVN (Figure 5(a)). As assessed by IHC, CCL₄-mediated infiltration into the liver of macrophages (F4/80 +), monocytes (Ly6C+) and T cells (CD45+) was suppressed by EVN treatment (Figure 5(b)), consistent with

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**Figure 4.** Normalization by EVN of hepatic gene expression or serum components during CCl₄- or TAA-induced liver fibrosis in vivo. Male TG CCN2-EGFP mice were administered oil (1500 μl/kg) or CCl4 (175 μl in 1325 μl corn oil/kg) i.p. for 5 weeks, with or without EVN (40 μg/g; i.p.) every other day over the last 2 weeks. Animals were then sacrificed and examined by (a) qRT-PCR of hepatic mRNA for expression of CCN2, αSMA, or collagen α1(I) (n = 4 independent experiments performed in triplicate. *p < 0.01 versus no treatment)) or (b) IHC of liver sections for the presence of caspase 3 or αSMA (left panel) or Ki67 or albumin (right panel) (typical staining from 5 independent experiments). Serum from blood collected at the time of sacrifice was (c) assayed for ALT or AST or (d) subjected to NTA to determine EV frequency. Male FVB mice received TAA (i.p. 200μg/g; q.o.d.) for 6 wks with or without daily i.p. administration of EVN (40μg/g; isolated from serum of normal FVB mice) for 6 days during the last week after which (e) hepatic RNA underwent qRT-PCR for fibrosis-related gene expression in livers or (f) liver sections were stained with H and E or with anti-collagen α1 (orange) and DAPI (blue). n = 5 independent experiments performed in triplicate. *p < 0.01 versus no treatment. Scale bar: 20 μm.
the EVN-mediated reduction of CCl₄-induced infiltrating cells assessed by H and E staining (Figure 3(c)). CCl₄-induced hepatic interleukin (IL)-1β, monocyte chemotactic protein-1 (MCP-1) or tumour necrosis factor alpha (TNFα) which are produced by injured hepatocytes and infiltrating cells were also suppressed by EVN as assessed by IHC (Figure 5(c)). Protein array analysis showed that EVN attenuated 5 of the same cytokines and chemokines in liver tissue (Figure 5(d)) as in the circulation (Figure 5(a)) (IFN-γ, IL-2, IL-4, IL-10, TNFα) and also that 22 additional components were attenuated by EVN in fibrotic mice (Table 2). EVN treatment of non-treated or oil-treated mice did not cause circulating cytokine levels to be altered (Figure 5(a)), nor as assessed by staining did it cause any infiltration of inflammatory cells (Figure 5(b) and (c)) or expression of pro-inflammatory cytokines in the liver (Figure 5(b, c)). As assessed by protein array, there appeared to be slight changes in hepatic cytokine or chemokine expression in control mice exposed to EVN but this has yet to be validated by independent analysis and in
any case it was not associated with tissue damage or pathology. Thus, while EVN exerted profound anti-inflammatory actions in fibrotic mice, they did not elicit overt inflammatory reactions in control mice.

**E VN are tropic for HSC or hepatocytes and show enhanced cellular binding after injury**

To identify target cells in the liver, PKH26-stained EVN were injected once i.v. into mice at the end of a 5-week course of oil or CCl₄ administration. Direct imaging of the livers after 4 h showed the presence of hepatic PKH26 fluorescence, for which the staining intensity was much greater in fibrotic livers than in control livers (Figure 6(a)). This finding was also evident in liver tissue sections (Figure 6(b)) and was attributable to the association of PKH26 with hepatocytes or HSC as shown by the presence of PKH26 staining in each cell type when isolated from the livers of control mice and by marked increase in PKH26 staining intensity in each cell type when they were isolated from CCl₄-treated mice (Figure 6(c, d)). To corroborate these findings, EV binding studies were performed on primary cultures of HSC or hepatocytes. *In vitro* binding of PKH26-stained EVN to P3 (activated) HSC was ~6-fold higher than to Day 1 (quiescent) HSC, while EVN binding to primary D2 hepatocytes was ~5-fold higher after *in vitro* exposure of the cells to CCl₄ (Figure 6(e)).

**Attenuation of hepatocyte injury or HSC activation in vitro by EVN**

The direct binding of EVN to hepatocytes or HSC *in vivo* or *in vitro* supported the possibility that EVN was beneficial for each cell type during fibrosing injury. To test this, *in vitro* experiments were first conducted on AML12 mouse hepatocytes that had been exposed to CCl₄ or ethanol for 48 h (Figure 7(a, b)). Whereas hepatocyte proliferation was decreased by CCl₄ or ethanol, this outcome was reversed in cells to which EVN were added for the final 24 h (Figure 7(a, b)). Moreover,

| Molecule | Array location (Row:Dots) | Control | ExoN | CCl₄ | ExoN + CCl₄ | % suppression by ExoN of CCl₄ response |
|----------|---------------------------|---------|------|------|-------------|---------------------------------------|
| BLC      | 1:1–2                     | 1       | 1.12 | 1.90 | 1.05        | 100                                   |
| CS/C5a   | 1:3–4                     | 1       | 1.23 | 1.66 | 1.21        | 100                                   |
| G-CSF    | 1:5–6                     | 1       | 1.15 | 1.17 | 1.17        | -                                     |
| GM-CSF   | 1:7–8                     | 1       | 1.15 | 1.17 | 1.25        | -                                     |
| I-309    | 1:9–10                    | 1       | 1.14 | 1.42 | 1.25        | 61                                    |
| Eotaxin  | 1:11–12                   | 1       | 1.16 | 1.37 | 1.20        | 81                                    |
| sICAM-1  | 1:13–14                   | 1       | 1.01 | 1.41 | 1.12        | 73                                    |
| IFN-γ    | 1:15–16                   | 1       | 1.13 | 1.61 | 1.15        | 96                                    |
| IL-1α    | 1:17–18                   | 1       | 1.13 | 1.38 | 1.49        | -                                     |
| IL-1β    | 1:19–20                   | 1       | 1.14 | 1.18 | 1.22        | -                                     |
| IL-1α    | 1:21–22                   | 1       | 0.78 | 2.95 | 0.67        | 100                                   |
| IL-2     | 1:23–24                   | 1       | 1.11 | 1.88 | 1.29        | 77                                    |
| IL-3     | 1:2–2                     | 1       | 1.14 | 1.25 | 1.11        | 100                                   |
| IL-4     | 2–4                       | 1       | 1.13 | 1.26 | 1.13        | 100                                   |
| IL-5     | 2–5                       | 1       | 1.15 | 1.17 | 1.14        | -                                     |
| IL-6     | 2–7                       | 1       | 1.14 | 1.16 | 1.14        | -                                     |
| IL-7     | 2–9                       | 1       | 1.14 | 1.29 | 1.27        | -                                     |
| IL-10    | 2–11–12                   | 1       | 1.15 | 1.34 | 1.13        | 100                                   |
| IL-13    | 2–13–14                   | 1       | 0.99 | 2.08 | 1.59        | 45                                    |
| IL-12p70 | 2–15–16                   | 1       | 1.14 | 1.19 | 1.17        | 40                                    |
| IL-16    | 2–17–18                   | 1       | 1.16 | 1.82 | 1.42        | 61                                    |
| IL-17    | 2–19–20                   | 1       | 1.12 | 1.30 | 1.28        | -                                     |
| IL-23    | 2–21–22                   | 1       | 1.02 | 1.78 | 1.22        | 74                                    |
| IL-27    | 2–23–24                   | 1       | 0.98 | 1.34 | 1.09        | 70                                    |
| IP-10    | 3–1–2                     | 1       | 1.13 | 1.32 | 1.10        | 100                                   |
| I-TAC    | 3–3                       | 1       | 1.13 | 1.30 | 1.12        | 100                                   |
| KC       | 3–5–6                     | 1       | 1.14 | 1.33 | 1.22        | 58                                    |
| M-CSF    | 3–7–8                     | 1       | 1.15 | 1.22 | 1.26        | -                                     |
| JE       | 3–9–10                    | 1       | 1.15 | 1.20 | 1.20        | -                                     |
| MCP-5    | 3–11–12                   | 1       | 1.14 | 1.23 | 1.18        | 56                                    |
| MIG      | 3–13–14                   | 1       | 1.14 | 1.44 | 1.24        | 50                                    |
| MMP-1α   | 3–15–16                   | 1       | 1.13 | 1.20 | 1.18        | 29                                    |
| MMP-1β   | 3–17–18                   | 1       | 1.13 | 1.22 | 1.25        | -                                     |
| MMP-2    | 3–19–20                   | 1       | 1.13 | 1.18 | 1.18        | -                                     |
| RANTES   | 3–21–22                   | 1       | 1.09 | 1.36 | 1.14        | 49                                    |
| SDF-1    | 3–23–24                   | 1       | 0.79 | 2.44 | 1.93        | 31                                    |
| TARC     | 4–1–2                     | 1       | 1.12 | 1.46 | 1.15        | 92                                    |
| TIMP-1   | 4–3–4                     | 1       | 1.07 | 1.54 | 1.06        | 100                                   |
| TNF-α    | 4–5–6                     | 1       | 1.12 | 1.85 | 1.14        | 97                                    |
| TREM-1   | 4–7–8                     | 1       | 1.14 | 1.18 | 1.18        | -                                     |
release of AST or ALT after exposure of hepatocytes to CCl_4 in vitro reverted to baseline levels after exposure of the cells to EVN (Figure 7(c, d)). When tested on activated mouse HSC in vitro, EVN reduced the expression of collagen α1(I), αSMA or CCN2 and the rate of cell proliferation (Figure 7(e, f)). Thus in vitro, EVN drive proliferation in injured hepatocytes and suppress fibrogenesis and proliferation in activated HSC, consistent with the outcomes seen in the livers of EVN-treated fibrotic mice. In contrast to the inhibitory effect of EVN, HSC proliferation trended upwards in response to EVF, but this was not statistically significant (Figure 7(f)).

Identification of therapeutic miRs in EVN

Since miRs exert their effects over multiple targets and across many signalling pathways and exosomal miRs have been shown previously to regulate activated HSC [16,17,19,26], we focussed on the identity of candidate therapeutic miRs in EVN. Differential miRnome profiling was performed to identify EV miRs that were, as compared to EVN, the most differentially suppressed in EVF but not significantly changed in serum EV from oil-treated animals. The results of this analysis revealed that miR-34c-3p, -151-3p, -483-5p, -532-5p and -687 were among the candidate miRs identified (Figure 8(a)). When independently assessed by RT-PCR in additional groups of control or fibrotic mice, each of these miRs was confirmed to be expressed at higher levels in serum EV from control versus fibrotic mice (Figure 8(b)). Mimics of each miR attenuated expression of collagen α1(I), αSMA or CCN2 when tested on D9 mouse HSC (Figure 8(c)), while Smad3 expression in HSC or hepatocytes was reduced by either EVN or a miR-532 mimic (Figure 8(d)). To establish the role of these miRs as functional constituents of EVN, the responses of target HSC (activated) or hepatocytes (CCl_4-injured) were...
Effects of EVN on HSC or hepatocytes

Male and female human blood donors did not significantly differ in age (males: 25.5 ± 1.5 years; females: 25.5 ± 2.3 years). Based on TEM, NTA and Western blot, human serum EVs were highly comparable within and between gender, except that those from females were ~20% larger than those from males (164 ± 10 nm vs. 137 ± 8 nm) (Figure 9(a, b)). Even so, as determined by individual analysis of each human serum sample, there was clear heterogeneity in their respective mean size, size-range and relative expression of CD81 or flotillin (Supplemental Figure 1(a–d)). Pooled human serum EVs demonstrated dose-dependent binding to LX-2 cells (a human activated HSC line) (Figure 9(c)) and caused an attenuation of collagen α1(I) mRNA expression (Figure 9(d)) and of TGF-β-induced αSMA protein production (Figure 9(e)). The biological activity of serum EVs from individual donors was again heterogeneous, with EVs from 7 of 9 females or from 6 of 9 males causing an inhibition of collagen expression of between 25% and 60% (Supplemental Figure 1(e, f)). Although miR-687 was not detected, expression of miR-34c-3p, -151-3p, -483-5p and -532-5p was higher in EVs from serum of normal human subjects as compared to those of patients with F3/4 fibrosis (Figure 9(f)).

Discussion

There is growing interest in understanding the function of EVs in hepatic intercellular communication and their potential as carriers of biomarkers to aid disease diagnosis and prognosis [7,27]. While analysis of cargo molecules in circulating EVs has begun to identify potential candidate markers for disease assessment in chronic liver diseases [9–14], our findings for EVN provide an important distinction from these prior studies in that they show that EVs from healthy individuals have inherent therapeutic properties that drive anti-fibrotic outcomes in in vitro or in vivo models. EV treatment resulted in gender-independent therapeutic effects in three strains of mice using in vivo fibrogenesis (short-term CCl₄ or fibrosis models (chronic CCl₄ or TAA). HSC were found to be among the principal cells involved, with EVN demonstrating greater binding to activated HSC than to quiescent HSC and exerting suppressive effects on activated HSC function, including fibrogenesis and proliferation. As compared to mouse serum EVs, human serum EVs exhibited comparable biophysical and biochemical properties (TEM, NTA, Western blot) and, importantly, they similarly attenuated fibrogenic- and activation-associated gene expression in human HSC and also contained the same differentially expressed miRs between normal and fibrotic subjects. This suggests the existence of evolutionarily conserved actions of serum EVs in regulating HSC, and
possibly some of the miR mediators involved, although analysis of other hepatic fibrosis patient populations (e.g. non-alcoholic steatohepatitis, alcoholic liver disease, hepatitis C, etc.) in addition to the fibrotic HBV patients studied here will be necessary to fully answer this question. Additionally, we showed that hepatocytes are also a target of EVN and that, following injury in vitro or in vivo, EVN bind more strongly to hepatocytes, reduce cellular damage and drive proliferation. The therapeutic effects of EVN in vivo were further manifested by a decreased incidence in proinflammatory cells in the liver and by normalization of the levels of circulating proinflammatory cytokines. Importantly, as assessed in normal mice, EVN administration did not
Figure 9. Characterization and actions of human serum EVs. (a) TEM (upper panel; scale bar: 100 nm; representative images are shown), NTA (lower panel) and Western blot analysis (lower panel, inset) of EVs purified by ultracentrifugation of serum of normal male or female human blood. Data are from individual donors and are representative of each gender (n = 9 males, 9 females). (b) Size (upper panel) and concentration (lower panel) of serum EVs from individual NTA analysis of all human donors (n = 18), with data grouped by gender (n = 9 per group). (c) LX-2 cells were incubated for 16 h with purified human serum EVs that had been labelled with PKH26 dye. Cell-associated PKH26 was determined by fluorescence measurement in cell lysates. n = 3 independent experiments performed in triplicate. *P < 0.01 versus no treatment. (d) Suppression of collagen α1(I) gene expression in LX-2 cells that were serum-starved for 24 h and then treated for 24 h with 5 × 10^8 particles/ml pooled human serum EVs from healthy male or female donors. n = 9 independent experiments performed in triplicate. *P < 0.01 versus control. (e) αSMA IHC in LX-2 cells that were serum-starved for 24 h and then incubated in the presence of TGFβ for 36 h, with or without human serum EVs for the last 24 h. Images are representative of five independent experiments. Scale bar: 20 μm. (f) RT-PCR of serum EV miRs in control subjects versus F3/F4 fibrosis patients (*P < 0.05).
cause hepatic inflammation or histopathology, nor did it result in overt alterations in cytokines or inflammatory mediators in the circulation or liver. The absence of pathophysiologica}
that limits aberrant fibrogenesis at sites of injury in otherwise healthy individuals. Since unbiased array analysis was an effective means of identifying EV miRs with novel anti-fibrotic actions in the liver, similar strategies could be used in the future to identify a more complete slate of anti-fibrotic components in EVN that might also include mRNAs or proteins. Given the complexities of the many EV subpopulations in serum and the multitude of producer and target cells, future studies will also need to address whether EVN represents a mixture of EV subpopulations from a variety of donor cells (one of which may be hepatocytes as discussed above), each possibly carrying distinct anti-fibrotic moieties.

Recent studies have begun to identify an EV hepatic cellular communication network which contributes to hepatic pathophysiology via EV-mediated functional reprogramming in target cells [7]. Transmission of hepatitis C virus between hepatocytes is mediated by liberation of virus-containing EVs from infected cells [54] while EV communication between hepatocarcinoma cells drives tumour progression and chemoresistance [55,56]. EVs obtained from hepatocytes during non-alcoholic fatty liver disease, non-alcoholic steatohepatitis or alcoholic hepatitis drive activation or chemotaxis in macrophages as well as features of angiogenesis or activation in, respectively, endothelial cells or HSC [26,57–61]. Migratory responses and expression of activation markers in HSC are also stimulated by exosomes from, respectively, endothelial cells or other activated HSC [62,63]. In these prior studies, the observed responses were attributed to the uptake into the recipient cells and subsequent action of a variety of EV cargo molecules including CXCL10 [58] S1P [59,64], TRAIL [32], vanin-1 [60], CD40L [57], SK1/2[59,64], CCN2[25] or miRs [26]. An important feature of many of these findings is that the pathophysiology-related pathways were driven by EVs derived from cells that were injured (e.g. hepatocytes) or that were phenotypically or functionally altered in an injury-related manner (e.g. activated HSC) whereas the therapeutic EVs identified in our study were from healthy individuals. Indeed, we showed that, unlike EVN, EVF do not suppress proliferation or expression of fibrosis- or activation-related genes in HSC. Since inflammatory pathways in the liver have been linked to EV communication between hepatocytes and monocytes [65], an interesting area for future study will be to determine whether the therapeutic actions of EVN in fibrosis are in part attributable to the action of EV miRs and/or other EV constituents on pro-inflammatory gene expression and regulation of inflammatory cell function.

In conclusion, serum EVs from healthy donors have anti-fibrogenic properties that are attributable, at least in part, to specific miR constituents that have therapeutic actions in activated HSC or injured hepatocytes. Further studies of these miRs, other therapeutic molecules in the EV payload and EV homing will help to establish mechanisms of EV-mediated innate fibrosis resistance and the utility of leveraging these components for developing new anti-fibrotic strategies.

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Disclosure statement

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