Exosomes are cell-derived extracellular vesicles thought to promote intercellular communication by delivering specific content to target cells. The aim of this study was to test the hypothesis that endothelial cell (EC)-derived exosomes could regulate the phenotype of hepatic stellate cells (HSCs). Initial microarray studies showed that fibroblast growth factor 2 induced a 2.4-fold increase in mRNA levels of sphingosine kinase 1 (SK1). Exosomes derived from an SK1-overexpressing EC line increased HSC migration 3.2-fold. Migration was not conferred by the dominant negative SK1 exosome. Incubation of HSCs with exosomes was also associated with an 8.3-fold increase in phosphorylation of AKT and 2.5-fold increase in migration. Exosomes were found to express the matrix protein and integrin ligand fibronectin (FN) by Western blot analysis and transmission electron microscopy. Blockade of the FN-integrin interaction with a CD29 neutralizing antibody or the RGD peptide attenuated exosome-induced HSC AKT phosphorylation and migration. Inhibition of endocytosis with transfection of Dyn2K44A, or the pharmacological inhibitor Dynasore attenuated exosome-induced HSC AKT phosphorylation and migration. Inhibition of endocytosis with transfection of dynamin siRNA, the dominant negative dynamin GTPase construct Dyn2K44A, or the pharmacological inhibitor Dynasore significantly attenuated exosome-induced AKT phosphorylation and migration. SK1 levels were increased in serum exosomes derived from mice with experimental liver fibrosis, and SK1 mRNA levels were up-regulated 2.5-fold in human liver cirrhosis patient samples. Finally, S1PR2 inhibition protected mice from CCl4-induced liver fibrosis. Therefore, EC-derived SK1-containing exosomes regulate HSC signaling and migration through FN-integrin-dependent exosome adherence and dynamin-dependent exosome internalization. These findings advance our understanding of EC/HSC cross-talk and identify exosomes as a potential target to attenuate pathobiology signals.

Liver fibrosis is the excessive accumulation of extracellular matrix proteins, including collagen I, that occurs in various forms of chronic liver diseases. Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation. Although the pathogenesis of liver fibrosis is not fully defined, the activation of hepatic stellate cells (HSCs) into a myofibroblastic phenotype is recognized as a cardinal pathogenic step in the development of this disease. One key component of HSC activation is enhanced migration, which allows HSCs to navigate to sites of fibrogenesis. In many circumstances, HSC migration is governed by molecules released from neighboring endothelial cells (ECs). The molecular mechanisms that define this process remain incompletely defined.

Exosomes are extracellular membrane vesicles that are produced in multivesicular bodies or at the plasma membrane. They have an average diameter of 40–100 nm, a characteristic cup-shaped morphology and sediment at 1.13–1.19 g/ml, and fibronectin (FN) binding with a characteristic density (1.13–1.19 g/ml), and a cup-shaped morphology and sediment at 100,000 X g (8). Exosomes are enriched with endothelial cell (EC) intercellular communication by delivering specific content to target cells. The aim of this study was to test the hypothesis that EC-derived exosomes regulate pathological HSC migration during liver fibrosis. The experimental results from this study provide evidence that exosome-induced HSC migration is dependent first on exosome adhesion, which is mediated by exosome fibronectin (FN) binding with α5β1-integrin on target cells. Second, adhesion facilitates exosome entry into the target cell through dynamin-dependent endocytosis. These steps are requisite for signal activation and ensuing migration. At the molecular level, we identified the lipid enzyme sphingosine kinase 1 as a potential target to attenuate pathobiology signals.
(SK1) as a critical mediator of exosome actions on HSCs. Both SK1 and its product sphingosine 1-phosphate (S1P) are present within the exosome and are required for chemotactic effects. The experimental results extend our understanding of the mechanisms controlling exosome regulation of HSCs. More broadly, the work extends our understanding of paracrine signaling and also lays the theoretical foundation for therapies targeting exosomes in the treatment of liver pathobiology such as fibrosis.

**Experimental Procedures**

**Cell Culture and Viral Transfection**—The LX-2 human HSC cell line, the primary human HSC cell line (hHSC), and immortalized liver sinusoidal ECs (TSECs) (6) were grown in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin (Gibco). Human umbilical vein endothelial cells were grown with endothelial culture media with 10% serum and 1% endothelial growth supplement. Liver hepatocellular carcinoma cells (HepG2) were routinely maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin. The human macrophage line (THP-1) was cultured in RPMI 1640 medium with 10% fetal bovine serum and 1% penicillin/streptomycin. The culture medium used for exosome isolation was prepared with exosome free-FBS as described previously (8). Adenoviral vectors were generated through the Iowa Vector Core and encoded dynamin-2 K44A or a LacZ control. Cells were incubated for 12 h with 0.1% albumin/PBS with adenoviruses (multiplicity of infection of 50), which achieved a transduction efficiency approaching 90% with minimal toxicity. Lentivirus was generated by using 293T cells. Adenoviral and lentiviral transduction were performed as described previously (4, 8). All cell lines were maintained under standard tissue culture conditions (37 °C, 5% CO2 incubator).

**Isolation of Mouse Liver Endothelial Cells**—Liver endothelial cells were isolated from whole livers of healthy mice and mice subjected to CCl4-induced fibrosis by mechanical disruption, subjected to CCl4-induced fibrosis by mechanical disruption, cells were isolated from whole livers of healthy mice and mice subjected to CCl4-induced fibrosis by mechanical disruption, and analyzed by NanoSight software (NTA 2.3.5 B16) (11, 12). Briefly, liver tissue was perfused, harvested, dissected, minced, and digested in a collagenase buffer and incubated with immunomagnetic Dynabeads (Dynal) coated with rat anti-mouse CD146 (BD Biosciences), an endothelial marker, for 1 h at room temperature (18, 19). Cells were separated with a magnet and plated on collagen I-coated dishes. Viability was >90% by trypan blue staining, and purity was >95% by staining for CD31. Cells were grown in EC growth medium containing 5% fetal bovine serum, 2% endothelial cell growth supplement, and 1% penicillin/streptomycin (ScienCell, San Diego, CA) and maintained under standard tissue culture conditions (37 °C, 5% CO2 incubator).

**Site-directed Mutagenesis and Generation of Stable Cell Lines**—Site-directed mutagenesis was performed according to the protocol of the manufacturer (Agilent Technology, Santa Clara, CA). cDNA encoding full-length wild-type hSK1-pCW4/5 was purchased from Addgene (Cambridge, MA). SK1 dominant negative and constitutively active mutants with an Asp-81 to Ala (D81A) and Gly-113 to Asp (G113A) (20, 21) mutation, respectively, were generated by performing PCR using the respective oligonucleotides 5′-CTCTGGTGGTCA-TGTCTGGAAACGGGCATGACGGTT-3′ and 5′-CACCTGGTCATTACGGCTTTCCAGACATGACCAGCAGAG-3′ (for the D81A mutant) and 5′-GTAGCTCCCGACAGGCTTGGGCAAGCCGCTGCTGGAGGCTAC-3′ and 5′-AAGGAAGCTGCCAGCGGTTGGCAGAGCCGCTGCTGGAGGCTAC-3′ (for the G113A mutant). Mutations were verified by DNA sequencing. Lentivirus was generated using 293T cells and used to transduce TSECs and establish stable cell lines that expressed SK1 wild-type and SK1 D81A and G113A mutants. Exosomes were isolated from the conditioned media generated from cell lines.

**Exosome Purification and Fluorescent Labeling**—Exosomes from de-identified patient serum, from cell culture supernatant of TSECs, and from a pool of murine serum (~300 μL) were purified as described previously (6, 8, 22). The protein concentration contained in each exosome pellet was quantified using a Bradford assay (Bio-Rad) (9, 23). Experiments were performed using 50 μg of exosomes unless indicated otherwise. Isolated exosomes were characterized by Western blot (WB), nanoparticle tracking analysis, and electron microscopy immunogold labeling. Precipitated exosomes were labeled in some experiments with the green fluorescent linker PKH67 (Sigma-Aldrich) according to the instructions of the manufacturer.

**Immunofluorescence, Confocal Microscopy, and Image Quantification**—PKH67-labeled exosomes were incubated with HSCs for different periods of time. HSCs were first fixed with 4% paraformaldehyde followed by permeabilization with 0.1% Triton X-100. Cells were incubated with primary antibodies (early endosome antigen (EEA), 1:200, catalog no. 610456; LAMP1, 1:200, catalog no. sc20011) overnight at 4 °C, and the appropriate Alexa Fluor-conjugated second antibodies (Invitrogen, Thermo Fisher Scientific) were used for secondary detection. Cell nuclei were counterstained with DAPI (1:2000). Fluorescence confocal microscopy was performed using an LSM 510 laser-scanning microscope (Carl Zeiss, Jena, Germany) using a ×63 or ×40 lens. ImageJ (National Institutes of Health) was used to analyze the raw images. The images used in the figures are representative of trends observed in all images obtained. Co-localization images were created using the ImageJ co-localization plugin. The quantification shows the Pearson coefficients of co-localization between the green channel for the biotin exosome and red channel for EEA or LAMP1. More than six representative pictures in each group were selected for quantification and statistical analysis.

**Nanoparticle Tracking Analysis**—The presence, size distribution, and concentration of vesicles were assessed by nanoparticle tracking analysis using a NanoSight NS300 instrument (NanoSight Ltd., Amesbury, UK). Exosome samples were diluted with PBS at a range of concentration of 4–8 × 108 particles/ml. Each sample was loaded into a flow cell top plate using a syringe pump, and three videos of 30 s were recorded and analyzed by NanoSight software (NTA 2.3.5 B16) (11, 24, 25).

**Western Blot Analysis**—HSCs or liver tissue were lysed and prepared for WB analysis as described previously (26, 27). Immunoblot analysis was performed according to the protocol recommended for individual antibodies as listed in supplemental Table...
1. Immunoreactive bands were visualized using horseradish peroxidase-conjugated secondary antibody and an enhanced chemiluminescent system (Santa Cruz Biotechnology, Inc.). All experiments were performed in triplicate, and quantitation was done by densitometry.

**PCR Studies**—The RNasey kit (Qiagen) was used to extract total RNA from cells and mouse tissue according to the instructions of the manufacturer. 10 μg of mRNA was used for cDNA synthesis with dNTP and oligo primer using the SuperScripTM III (Invitrogen) first strand synthesis system for RT-PCR according to the protocol of the manufacturer. Real-Time PCR was performed with the same amount of cDNA in a total 25-μl volume reaction using IQ SYBR Green Mix (Bio-Rad) and the 7500 real-time PCR system (Applied Biosystems) according to the instructions of the manufacturer. Amplification of GAPDH and β-actin was performed in the same reaction for the respective samples as an internal control. Each experiment was done in triplicate. Primer sequences are listed in Supplemental Table 2.

**Transwell Migration Assays**—Exosomes, compounds (Dynasore, 10 μM; dimethylsphingosine (DMS), 10 nM; FTY720, 10 nM; and AKT inhibitor VIII, 25 nM), and FN-integrin binding neutralizing reagents (RGD, 20 nM; CD29, 0.2 μg/ml) were used to assess HSC migration as measured by Transwell assay. Briefly, 8-μm-pore polycarbonate filters (catalog no. 3422, Costar) were coated with 1% (w/v) collagen I (Sigma-Aldrich, St. Louis, MO). Cells (1 × 10⁶ cells in 100 μl/well) were suspended in serum-free DMEM. Exosomes and/or reagents were added to the lower chamber in the same culture medium.

After incubation at 37 °C for 24 h, migrated cells were stained by crystal violet (8, 28). The number of cells that migrated to the bottom side of the chamber was determined by counting the number of cell per field by light microscopy (×10 magnification).

**Transmission Electron Microscopy and Immunogold Labeling**—EM and immuno-EM analysis of vesicles were performed as described previously (8, 29). Briefly, the specimens were resuspended and fixed in 4% paraformaldehyde for 1 h. Subsequently, the samples were dehydrated on formvar/carbon-coated EM grids (5 μl on each grid) and dried for 25 min. The vesicle-coated grids were washed twice with PBS (15 min each) and twice with PBS/50 mM glycine (15 min) and blocked with 10% FBS/PBS (15 min). For the immunogold labeling with antibodies, blocked grids were transferred to a drop of the antibody (listed in Supplemental Table 1) and incubated for 1 h. The grids were then washed with 2% FBS/PBS for 5 × 3 min, incubated with specific gold-labeled secondary antibody (10 nm gold, goat-anti-rabbit IgG 10 nm gold, rabbit anti goat IgG 10 nm), diluted with 10% FBS/PBS in a ratio of 1:30 for 1 h, washed 5 × 3 min in 100-μl drops of 2% FBS/PBS, and then post-fixed with 1% glutaraldehyde for 5 min. After washing in 8 drops of distilled water, the grids were stained with a mixture of 4% uranyl acetate and 2% methylcellulose (1:9) and viewed for transmission electron microscopy using a JEOL 1400.

**Scanning Electron Microscopy**—Scanning electron microscopy was performed as described previously (8, 14). Samples were fixed in Trump fixative for 1 h or overnight at 4 °C. After washing three times with 0.1 M phosphate buffer, the specimens were post-fixed in 1% OsO₄/0.1 M phosphate buffer for 1 h. After washing with distilled water three times, they were dehydrated with a series of ethanol (50%, 70%, 95%, 100%, and 100%), dried with critical point drying, mounted and gold-paladium sputter-coated, and then viewed by Hitachi 4700.

**Sphingosine 1-Phosphate ELISA**—Sphingosine-1-phosphate ELISA (K-1900, Echelon Biosciences Inc.) was performed according to the instructions of the manufacturer.

**Biotinylation Assay for Plasma Membrane Integrin Activity**—Biotin-based assays were performed as described previously (22, 30). HSCs were fasted overnight and treated with exosome for 15 min. Subsequently, the cells were placed on ice and washed once with cold Dulbecco’s phosphate-buffered saline. Cell surface proteins were labeled with 1 mg/ml of EZ-Link sulfosuccinimidyl-6-((4-halo-1,2,3-triazol-1-yl)ethyl)amino)hexanoate (Pierce) in 1% (v/v) BSA-PBS for 30 min at 4 °C and then washed three times with PBS. Cell surface proteins were labeled with 1 mg/ml of EZ-Link sulfo-NHS-SMCC (Pierce) in 1% (v/v) BSA-PBS for 30 min at 4 °C and then washed three times with PBS. Cell surface proteins were labeled with 1 mg/ml of EZ-Link sulfo-NHS-SMCC (Pierce) in 1% (v/v) BSA-PBS for 30 min at 4 °C and then washed three times with PBS.
determined from 5 animals/group as described previously (North Carolina Animal Lab Test Center) (9).

Human Subject-derived Samples—The patient-derived serum was approved by the Ethics Commission of the Mayo Clinic (Institution Review Board no. 13-002715). Samples derived from study subjects were used for isolation, quantification of the exosome, and detection of the S1P level.

Statistical Analysis—Results are expressed as the mean of three or more independent experiments. Two-tailed Student’s t test or analysis of variance was used to test the statistical significance between groups as appropriate. p ≤ 0.05 was considered statistically significant.

Results

SK1 Is an EC-derived Exosome Protein—Because ECs generate a significant percentage of the serum pool of exosomes (32) and are anatomically situated near HSCs, we focused on ECs and their exosome production as a paracrine mechanism of HSC migration (33). First, we performed angiogenesis pathway-specific microarray studies to identify specific candidate EC genes for further study. Studies were performed with the FGF2 ligand because this molecule has been implicated in both exosome release and liver fibrosis (23, 34). The results showed that FGF2 induced a 2.4-fold increase in SK1 mRNA levels in ECs (Fig. 1A). To confirm the array results in vivo, we isolated ECs from control and cirrhotic mice and showed that SK1 mRNA was elevated during liver fibrosis (Fig. 1B).

In vitro, we treated ECs with TGFβ, PDGF, or FGF2 to emulate liver fibrosis conditions and showed that EC SK1 mRNA was up-regulated (Fig. 1C). SK1 is an enzyme that produces the HSC chemotactic factor S1P. Additionally, active SK1 enzyme has been detected in the extracellular environment, although not specifically within an exosome fraction (24, 25, 27, 35–37). We therefore focused on SK1-derived S1P as a potential pathway relevant to our model of EC-HSC interaction.
Exosome and HSC S1P-induced Migration

As an initial step to explore the hypothesis that SK1 might be delivered by exosomes, we isolated exosomes by differential centrifugation from two models: in vitro ECs and human serum, both of which provide abundant sources of exosomes with potential pathophysiological relevance (14, 38). The presence of exosomes from these models was confirmed using several criteria, including the mode of diameter approximating 84 ± 5 nm in serum-derived exosomes and 92 ± 4 nm in TSEC-derived exosomes, as assessed by nanoparticle tracking analysis (Fig. 1D); a classic cup shape and double membrane morphology, as assessed by transmission electron microscopy (Fig. 1E); immunogold positivity for the exosome markers CD81, TIR (26, 39), CD63, and SK1 (Fig. 1E); and WB detection of additional well characterized markers of exosomes (TSG101, TIR, CD63, and SK1) (Fig. 1F).

To explore the functionality of exosomal SK1, exosomes were first isolated from the conditioned media of TSECs after lentiviral overexpression of SK1 (6). SK1 mRNA levels in TSECs were up-regulated 13-fold after SK1 transduction compared with control transduction (Fig. 1G). Additionally, SK1 protein was increased significantly in exosomes derived from SK1-overexpressing TSECs compared with the control (Fig. 1H). We then examined the S1P level in isolated exosomes to determine whether SK1 in exosomes could produce S1P. SK1-overexpressing exosomes contained 2-fold more S1P compared with control exosomes. Human serum-derived exosomes contained SK1 as well (Fig. 1I). With these well characterized exosome systems in hand, we next explored the effects of these exosomes on HSC activation in an in vitro model system.

Exosomal SK1 Promotes AKT Activation and HSC Migration—Because SK1/S1P have been implicated previously in cirrhosis (29, 40), we examined the effect of SK1-containing exosomes on HSC migration, a key feature of HSC activation. Treatment of HSCs with serum-derived exosomes induced a prominent activation of AKT signaling within 15 min (Fig. 2A). This time point is consistent with previous publications (41), prompting us to use a 15-min time point for the remainder of our experiments examining AKT phosphorylation. In conjunction with the pAKT analysis, HSC migration was analyzed using a Transwell assay. Treatment of HSCs with serum-derived exosomes induced robust HSC migration compared with bovine serum albumin controls (Fig. 2B). We obtained similar results of exosome-induced pAKT signaling and migration when we performed the same sets of experiments using a different hHSC cell line (Fig. 2, C and D). Two other cell types in liver, hepatocytes (HepG2) and macrophages (THP-1), were treated with exosomes. However, no significant up-regulation of pAKT was detected (Fig. 2, E and F). These results provide evidence that exosomes preferentially induce HSC pAKT signaling in the liver. Furthermore, to confirm the role of AKT in exosome-induced HSC signaling and migration, we performed WB and Transwell assays with the presence of AKT inhibitor VIII, which inhibited exosome-induced AKT phosphorylation and migration (Fig. 2, G and H).

In parallel, Transwell Assay and WB were performed in the presence of EC-derived SK1-overexpressing exosomes or control exosomes. SK1-overexpressing exosomes increased the pAKT/tAKT ratio 2-fold and the HSC migration rate 3.2-fold compared with control exosomes (Fig. 2I,J). Additionally, the S1P level in EC-conditioned media was increased 7.8-fold after treatment with SK1-overexpressing exosomes (Fig. 2K).

To provide further evidence that SK1 was the primary mediator of exosome-induced HSC signaling, we incubated serum-derived exosomes with the SK1 inhibitor DMS or the S1P antagonist FTY720. Transwell assays were performed using vehicle-treated exosomes or DMS-treated (10 nM) or FTY720-treated (10 nM) exosomes. SK1 inhibition in DMS-treated exosomes and FTY720-treated exosomes abolished exosome-induced migration (Fig. 2L).

To further establish the role of SK1 activity in exosome function, exosomes were isolated from SK1-mutant, lentivirus-stable, transduced TSEC-conditioned media. SK1 mRNA levels in TSECs were up-regulated after SK1 WT, D81A dominant negative, or G113A constitutively active mutant construct transduction (Fig. 2M). CTRL, SK1, D81A, and G113A exosomes were isolated, and SK1 overexpression was confirmed by Western blot analysis (Fig. 2N). SK1 activity of WT and mutant exosomes was also confirmed by ELISA, showing that the active mutant increased S1P (Fig. 2O). An HSC Transwell assay was performed using exosomes from CTRL-, SK1-, D81A-, and G113A-transduced TSECs. Exosomes from G113A cells induced more HSC migration compared with SK1, whereas exosomes from D81A cells showed decreased HSC migration compared with SK1 WT cells (Fig. 2P). These results implicate exosomal SK1 as a mediator of HSC signal transduction and migration through the delivery of S1P.

FN-Integrin Interactions Contribute to Exosome Adhesion and Are Required for Exosome-induced HSC Signaling and Migration—Our finding that SK1-overexpressing exosomes promote HSC migration motivated further mechanistic evaluation. It has been postulated that the delivery of exosome components may require exosome adhesion and/or endocytosis (3, 5, 10, 24, 25, 33, 34). The adhesion of exosomes was characterized by morphology, size, and number. We hypothesized that adhesion between exosomes and HSC may require ligand/receptor interactions to facilitate chemotactic signaling. FN is a glycoprotein of the extracellular matrix that is secreted by EC during liver fibrosis (27, 35–37, 42). Indeed, exosomes contained FN, as detected by FN immunogold labeling (Fig. 3A), consistent with previous reports (40, 43). To confirm that the observed fibronectin was derived from the exosome-producing cell and not a contaminant from plasma, an antibody against cellular fibronectin was used. Immunoblotting of serum-derived exosomes revealed that the FN observed was indeed cellular FN rather than plasma FN (Fig. 3B). To examine whether FN on exosomes was able to activate integrin on HSCs, biotin was used to label the surface protein of HSC, and streptavidin-beads were used to pull down the protein labeled by biotin. β1-integrin was activated after exosome treatment, implicating exosomal FN in the process of integrin activation and adhesion of exosomes to HSCs (Fig. 3C). Other extracellular matrix (ECM) proteins have been reported within exosomes (44, 45). To examine whether other ECM proteins could be responsible for integrin activation by exosomes, we blotted lysed exosomes for collagen I, which was not detected in our serum-derived exosomes. This result was consistent with plasma exosome
proteomic profiling reports, in which FN is the predominant ECM protein expressed on exosomes that could contribute to FN-integrin interactions (46, 47). CD29 or RGD, both of which block integrin-FN binding, decreased the cell surface binding of exosomes (Fig. 3D). We utilized scanning electron microscopy, which provided us with direct visualization of adhesion on the basis of exosome morphology, size, and number. Exosome-induced AKT signaling and migration were also abolished by CD29 and RGD treatment (Fig. 3, E and F). These findings implicate exosome adhesion as a requisite step for exosome-induced signaling and HSC migration.

We next examined potential links between exosome adhesion and endocytosis because exosome internalization has been implicated previously in exosome-mediated signaling (48). PKH67-labeled exosomes were incubated with HSCs in the presence of CD29 or RGD for 4 h. In the presence of CD29 or RGD, exosome endocytosis was decreased by 30% and 50%, respectively (Fig. 3G). These findings suggest that exosome adhesion is a critical early step required for exosome endocytosis. These insights then led us examine whether the endocytosis events ultimately mediate exosome-induced signaling and migration as well.
Exosome Endocytosis and Signaling Are Dependent on Dynamin-2—We next sought to determine whether signaling and migration of HSCs induced by exosomes required their internalization. The endocytic pathway involves multiple mechanisms, including both clathrin- and caveolin-coated vesicles, both of which are mediated by dynamin-2 GTPase activity, which is implicated in vesicle scission (38). We therefore perturbed dynamin function and assessed the effects on our exosome signaling model. After transfection of siRNA against dynamin-2, exosome-induced increases in the pAKT/tAKT ratio and HSC migration were both attenuated markedly (Fig. 4, A and B). We confirmed that exosome endocytosis was decreased by ~50% in the dynamin-2 siRNA-transfected cells using a PKH labeling technique (Fig. 4C).

We subsequently focused on disruption of dynamin-2 function using additional approaches. HSCs were transduced with the dynamin-2 K44A dominant negative construct using an adenoviral construct containing a point mutation in the GTP binding element that prevents GTP hydrolysis (49). In control cells, exosomes activated AKT after just 5 min. However, in dynamin-2 K44A cells, activation was delayed, occurring at 30 min (Fig. 4D). Consistent with this observation, a pharmacologic approach using the dynamin-2 inhibitor Dynasore also attenuated exosome-mediated AKT activation. Furthermore, PKH labeling studies confirmed that the dynamin-2 K44A construct inhibited exosome internalization by 50% compared with control cells (Fig. 4E). To assess the destination of internalized exosomes, we labeled exosomes with PKH67 and detected the subcellular signal by immunofluorescence. We found colocalization of exosomes with early endosomes and, subsequently, their presence in lysosomes on the basis of double immunofluorescence staining using the early endosome

![FIGURE 3. FN-integrin interactions contribute to exosome adhesion and are required for exosome signaling and HSC migration. A, immunogold staining for FN was performed. Scale bar = 200 nm. B, WB analysis showing the expression of cellular FN (cFN), collagen I, and TSG101 in three different batches of serum-derived exosomes (Exo). HSCs treated with TGFβ served as a positive control. C, HSCs were subjected to a biotinylation assay of activated β1-integrin, followed streptavidin pulldown and WB analysis for HUTS4 and CD29, from the input portion, as a loading control (n = 3; *, p < 0.05). D, scanning electron microscopy was performed to evaluate the effect of FN-integrin-blocking reagents (CD29 and RGD) on exosome uptake by HSCs. Exosome adhesion to HSCs is highlighted. Quantification of exosome number per cell is shown (n = 6; *, p < 0.05; ns, not significant). E, HSC pretreated with RGD or CD29 were treated with exosomes for different times. Cell lysates were subjected to WB for pAKT and tAKT analyses (n = 3; *, p < 0.05). F, exosomes treated with RGD or CD29 were incubated with HSCs to measure migration by Transwell assay (n = 3; *, p < 0.05). Veh, vehicle. G, exosomes were stained with PKH67 for quantification analyses. Stained exosomes were quantified after incubation with HSCs pretreated with vehicle, CD29, or RGD (n = 3; *, p < 0.05).]
marker EEA and the lysosome marker LAMP1 (Pearson coefficients of colocalization, 0.32 and 0.36, respectively) (Fig. 4, F and G). Therefore, these data support a model in which exosome-dependent migration of HSC requires dynamin-2 GTPase-dependent endocytosis.

Exosomal SK1 Contributes to Liver Fibrosis—SK1/S1P pathway has been implicated previously in liver fibrogenesis. This prompted us to explore SK1 expression and S1P production in an in vivo murine model of liver fibrosis with a focus on exosome mechanisms. SK1 was expressed in normal liver and was up-regulated markedly in cirrhotic liver samples from humans (2.5-fold, $p < 0.05$) (Fig. 5A). Exosomes isolated from alcoholic hepatitis patient serum contained 1.5-fold more S1P compared with healthy donor serum (Fig. 5B). The same effect was demonstrated following chronic liver injury induced in mice by administration of the fibrogenic hepatotoxin CCl4 or by BDL. SK1 mRNA was increased markedly in both CCl4 and BDL models of fibrosis ($\sim 20$-fold, $p < 0.05$, Fig. 5C). Levels of the HSC activation and fibrosis markers smooth muscle actin and collagen I, respectively, were up-regulated in the CCl4 mouse model compared with mice administered olive oil (Fig. 5D). Aspartate aminotransferase and ALT levels were significantly greater in CCl4 mice compared with olive oil-treated control mice (Fig. 5E). Sirius Red, a marker of collagen I deposition, was elevated in CCl4 mice as well (Fig. 5F). Furthermore, the serum S1P level was also increased 1.5-fold in CCl4-treated mice (Fig. 5G). We then isolated exosomes from the serum pool of olive oil- and CCl4-treated mice or SHAM and BDL mice by

FIGURE 4. Exosome endocytosis and signaling are dependent on dynamin-2. A, HSCs were transfected with control and dynamin siRNA, and transfection efficiency was tested by WB with dynamin-2 antibody. Left panel, Dynamin-2 knockdown cells were treated with exosomes and analyzed for dynamin-2, pAKT, tAKT and GAPDH expression by WB. Right panel, WB densitometry ($n = 5$; *, $p < 0.05$). B, cells treated with control and dynamin-2 siRNA were incubated with exosomes in a Transwell assay. Quantification of number of cells per field is shown ($n = 3$; *, $p < 0.05$; ns, not significant). C, PKH67-labeled exosomes were incubated with HSCs and then imaged to quantify endocytosis ability ($n = 3$; *, $p < 0.05$). D, WB for pAKT and tAKT. Right panel, HSCs transfected with LacZ or dynamin-2 K44A were incubated with exosomes for different times ($n = 8$; *, $p < 0.05$). Left panel, HSCs pretreated with the control (DMSO) or Dynasore were incubated with exosomes for different times ($n = 8$; *, $p < 0.05$). Densitometry results for both analyses are shown under the WB. E, PKH67-labeled exosomes were incubated with Dynasore-treated HSCs or LacZ/dynamin-2 K44A-transfected cells and subjected to FACS. The fold-change of positive PKH67 cells is shown ($n = 3$; *, $p < 0.05$). F and G, left panels, HSCs were incubated with PKH67-labeled exosomes for 1, 4, and 12 h and subjected to double immunofluorescence for PKH-67-labeled exosomes (green) and EEA (red in F), or LAMP1 (red in G). Cell nuclei were stained by Hoechst (blue). Right panels, Pearson coefficients of colocalization between exosomes and EEA or LAMP1 were analyzed by ImageJ ($n = 3$; *, $p < 0.05$). The comparison is between 0 h and different time points.
ultracentrifugation. S1P levels were up-regulated in exosomes from CCl4-treated mice and BDL mice exosomes compared with olive oil-treated mice or SHAM-treated mice (1.4-fold and 2.5-fold, respectively) (Fig. 5H). The WB results indicated that SK1 was up-regulated in exosomes derived from CCl4-treated mice compared with the olive oil group (normalized by TSG101 and CD63 expression, Fig. 5I). Nanoparticle tracking analyses further demonstrated that CCl4-treated mouse serum contained higher number of exosomes than serum from olive oil-treated mice (Fig. 5J). Exosomes derived from CCl4-treated murine serum induced more HSC migration than those derived from olive oil-treated mouse serum (Fig. 5K). Overall, these findings indicate that exosomal SK1 may contribute to liver fibrosis in vivo.

**Blockade of S1PR2 Attenuates Liver Fibrosis in Vivo**—Because exosomes contained SK1 and were up-regulated in cirrhosis, we next examined whether administration of the S1PR2 inhibitor (JTE-013) resulted in attenuated fibrosis in the CCl4-induced fibrosis mice model. JTE-013 was administered intraperitoneally twice a week prior to each CCl4 injection. We then examined the effects of JTE-013 on fibrotic markers. After 3 weeks of CCl4 treatment, serum ALT levels (Fig. 6A), exosome number (Fig. 6B), collagen I mRNA expression (Fig. 6C), SK1 mRNA (Fig. 6D), Sirius Red staining (Fig. 6E), a-smooth muscle actin protein expression, and collagen I protein expression (Fig. 6F) in liver tissue were up-regulated markedly. However, JTE-013 administration resulted in a significant attenuation in these parameters compared with mice treated with CCl4 alone. These
results further support the key role of S1P/SK1 and exosomes in liver fibrosis in vivo.

Discussion

Although the pathogenesis of liver fibrosis is not fully defined, the activation of HSCs into myofibroblasts is recognized as a sentinel step in the development of this disease (3, 5). This study includes novel observations that advance our understanding of how exosomes can contribute to this process, including the following: exosomes transduce a SK1/S1P pathway that mediates HSC migration, exosomal FN engages HSC integrins to mediate exosome adhesion, and HSC dynamin-2 mediates exosome entry into HSCs and eventual signaling. These findings extend our current understanding of both exosome and HSC biology.

Exosomes are increasingly implicated in a variety of pathobiological conditions, and some prior studies of liver cirrhosis have been variable in outcome. For example, serum exosome numbers are increased in patients with liver disease (14). In vitro studies indicate that exosomes may up-regulate the expression of fibrolytic matrix metalloproteinase genes in HSCs (42, 43). In other studies, exosomes were capable of transducing Hedgehog signals (13). Microparticles have also been implicated in cirrhosis and portal hypertension, with evidence that such particles may contain the angiogenic molecule VEGF and other studies providing evidence that these particles can mediate hemodynamic disturbances associated with cirrhosis (50). Our study adds to these existing models not only by showing a role for exosomes in paracrine signaling relevant to cir-

FIGURE 6. Blockade of S1PR2 attenuates liver fibrosis in vivo. C57BL/6 mice were subjected to olive oil or CCl4 for 3 weeks. An S1PR2 inhibitor (JTE-013) was administered 1 week before CCl4 injection and 1 day before each CCL4 injection. A, levels of ALT were measured by serum biochemical analysis in olive oil control-, CCl4-, and CCL4 + JTE-013-treated mice (n = 6; *, p < 0.05). B, nanotrack analysis was used to analyze the total number of exosomes from the serum of olive oil-, CCl4-, and CCL4 + JTE-013-treated mice (n = 6; **, p < 0.05; ns, not significant). C and D, levels of HSC activation and fibrosis markers (collagen I and SK1) were analyzed by real-time PCR (n = 6; *, p < 0.05). E, liver samples from olive oil control-, CCl4-, and CCL4 + JTE-013-treated mice were subjected to WB for α-smooth muscle actin (αSMA) and collagen I quantification (n = 6; *, p < 0.05). F, Sirius Red staining of liver sections was performed in olive oil control-, CCL4-, and CCL4 + JTE-013-treated mice to assess tissue collagen levels. The -fold change of collagen I-positive areas is shown, and a representative micrograph is also shown (n = 6; *, p < 0.05). G, proposed model of exosome function in HSC migration. Liver ECs release SK1-containing exosomes. Exosomes engage with HSCs via FN-integrin-dependent adhesion and dynamin-dependent endocytosis. SK1 is delivered by exosomes to induce HSC signaling and migration.
rhosis but also through mechanistic studies that elucidate how exosomes act on target cells.

The SK1-S1P pathway has been implicated previously in liver fibrosis. Our in vivo studies are consistent with previous reports showing that inhibition of the SK1-S1P axis protects mice from liver fibrosis. Co-treatment with an SK1 inhibitor, FTY720, JTE-013 (S1PR2 inhibitor), or VPC23019 (S1P R1/3 inhibitor) protected mice from BDL and CCl_4-induced liver fibrosis (51, 52). S1PR2 KO mice were protected from BDL-induced and CCl_4-induced liver fibrosis (53, 54), although a number of salient details are lacking regarding how this effect is achieved. Fortuitously, we identified that SK1 was up-regulated in ECs after FGF2 stimulation using an unbiased microarray-based approach. Additionally, prior studies have shown that active SK1 could be released from cells, although the mechanism and vehicle of release were not identified (1, 24, 25, 29). Conceptually, the packaging of SK1 into exosomes could protect the enzyme from degradation and maintain its stability until reaching its target cell of action. Indeed, we were able to confirm such a model in this study because pharmacologic and molecular inhibition of exosomal SK1 failed to induce HSC migration. Furthermore, we showed that SK1 was increased in serum-derived exosomes of cirrhotic mice, linking our exosome biology to a pathobiological process. Therefore, our results uncover several new pieces of information regarding the mechanisms of how exosomal SK1 could contribute to paracrine S1P signaling and fibrogenesis.

The mechanisms by which exosomes reach and attach to target cells are not well understood. Here we show that exosomes contain FN, a canonical ligand for specific cell surface integrins, and that this interaction can mediate exosome adhesion to HSCs. Adhesion appears to be a prerequisite step for exosome signaling in target cells and for endocytic entry of exosomes into target cells. Interestingly, integrin recycling and activation may be regulated by engagement with exosomal FN. In a recent study (3, 55), human macrophage-derived exosomes suppressed EC migration and abolished collagen I-induced ERK signaling pathways by blocking internalized integrin recycling. Therefore, sequential and reciprocal interactions between exosome adhesion and internalization may occur through the integrin endocytic recycling pathway.

Endocytosis may occur through multiple pathways, including phagocytosis, macropinocytosis, and clathrin- and caveola-mediated endocytosis (8, 56). In prior studies, the mechanism of exosome uptake has varied, and data exists to support each of these pathways (10, 12, 14, 57–60). In this study, exosome endocytosis was mediated by dynamin because knockdown or inhibition of this protein attenuated exosome uptake and associated signaling. Therefore, this work supports the hypothesis that exosome endocytosis is required for its signaling activity.

In summary, exosomal SK1 regulates HSC signaling and migration. FN-integrin induced adhesion between exosomes and HSC as well as subsequent dynamin-2 dependent exosome endocytosis are both required for signaling (Fig. 6G). Murine and human studies also support this model under pathobiological conditions. In total, these results enhance our understanding of exosome biology, paracrine vascular signaling, and fibrogenesis.

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References

1. Bataller, R., and Brenner, D. A. (2005) Liver fibrosis. J. Clin. Invest. 115, 209–218
2. Cao, Y., Szabolcs, A., Dutta, S. K., Yaqoob, U., Jagavelu, K., Wang, L., Leof, E. B., Urrutia, R. A., Shah, V. H., and Mukhopadhyay, D. (2010) Neuruplin-1 mediates divergent R-Smad signaling and the myofibroblast phenotype. J. Biol. Chem. 285, 31840–31848
3. Hernandez-Gea, V., and Friedman, S. L. (2011) Pathogenesis of liver fibrosis. Annu. Rev. Pathol. 6, 425–456
4. Cao, S., Yaqoob, U., Das, A., Shergill, U., Jagavelu, K., Huebert, R. C., Routray, C., Abdelmoneim, S., Vasdev, M., Leof, E., Charlton, M., Watts, R. J., Mukhopadhyay, D., and Shah, V. H. (2010) Neuruplin-1 promotes cirrhosis of the rodent and human liver by enhancing PDGF/TGF-β signaling in hepatic stellate cells. J. Clin. Invest. 120, 2379–2394
5. Moreira, R. K. (2007) Hepatic stellate cells and liver fibrosis. Arch. Pathol. Lab. Med. 131, 1728–1734
6. Huebert, R. C., Jagavelu, K., Liebl, A. F., Huang, B. Q., Splinter, P. L., LaRusso, N. F., Urrutia, R. A., and Shah, V. H. (2010) Immortalized liver endothelial cells: a cell culture model for studies of motility and angiogenesis. Lab. Invest. 90, 1770–1781
7. Kang, N., Gores, G. J., and Shah, V. H. (2011) Hepatic stellate cells: partners in crime for liver metastases? Hepatology 54, 707–713
8. Thery, C., Amigorena, S., Raopo, G., and Clayton, A. (2006) Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr. Protoc. Cell Biol. Unit 3.22
9. Yang, L., Kwon, J., Gajdos, G. B., Ordog, T., Brekken, R. A., Mukhopadhyay, D., Schuppan, D., Bi, Y., Simonetto, D., and Shah, V. H. (2014) Vascular endothelial growth factor promotes fibrosis resolution and repair in mice. Gastroenterology 146, 1339–1350.e1
10. Raopo, G., and Stoorvogel, W. (2013) Extracellular vesicles: exosomes, microvesicles, and friends. J. Cell Biol. 200, 373–383
11. Arendt, B. K., Walters, D. K., Wu, X., Tscharner, R. C., and Jelinek, D. F. (2014) Multiple myeloma cell-derived microvesicles are enriched in CD147 expression and enhance tumor cell proliferation. Oncotarget 5, 5686–5699
12. Masyuk, A. I., Masyuk, T. V., and Larusso, N. F. (2013) Exosomes in the pathogenesis, diagnostics and therapeutics of liver diseases. J. Hepatol. 59, 621–626
13. Watek, R. P., Yang, L., Liu, R., Jung, Y., Omenetti, A., Syn, W. K., Choi, S. S., Cheong, Y., Fearing, C. M., Agboola, K. M., Chen, W., and Diehl, A. M. (2009) Liver cell-derived microparticles activate hedgehog signaling and alter gene expression in hepatic endothelial cells. Gastroenterology 136, 320–330.e2
14. Lemoinne, S., Thabut, D., Housset, C., Moreau, R., Valla, D., Boulanger, C. M., and Rautou, P. E. (2014) The emerging roles of microvesicles in liver diseases. Nat. Rev. Gastroenterol. Hepatol. 11, 350–361
15. Huebert, R. C., Vasdev, M., Shergill, U., Das, A., Huang, B., Charlton, M., LaRusso, N. F., and Shah, V. (2010) Aquaporin-1 facilitates angiogenic invasion in the pathologic neovasculature that accompanies cirrhosis. Hepatology 52, 238–248
16. Yaqoob, U., Jagavelu, K., Shergill, U., de Assuncao, T., Cao, S., and Shah, V. H. (2014) FGF21 promotes endothelial cell angiogenesis through a dynamin-2 and Rab5 dependent pathway. PLoS ONE 9, e98130
17. Lalor, P. F., Edwards, S., McNab, G., Salmi, M., Jalkanen, S., and Adams, D. H. (2002) Vascular adhesion protein-1 mediates adhesion and transmigration of lymphocytes on human hepatic endothelial cells. *J. Immunol.* 169, 983–992

18. Schrage, A., Loddenkemper, C., Erben, U., Lauer, U., Hausdorf, G., Jung- blut, P. R., Johnson, J., Knolle, P. A., Zeitz, M., Hamann, A., and Klugewitz, K. (2008) Murine CD146 is widely expressed on endothelial cells and is recognized by the monoclonal antibody ME-9F1. *Histochem. Cell Biol.* 129, 441–451

19. van Beijnum, J. R., Rousch, M., Castermans, K., van der Linden, E., and Griffioen, A. W. (2008) Isolation of endothelial cells from fresh tissues. *Nat. Protoc.* 3, 1085–1091

20. Wang, Z., Min, X., Xiao, S. H., Johnstone, S., Romanow, W., Meininger, D., Hu, L., Liu, J., Dai, J., An, S., Thibault, S., and Walker, N. (2013) Molecular basis of sphingosine kinase 1 substrate recognition and catalysis. *Structure* 21, 798–809

21. Pitson, S. M., Moretti, P. A., Zebol, J. R., Vadas, M. A., D’Andrea, R. J., and Wattenberg, B. W. (2001) A point mutant of human sphingosine kinase 1 with increased catalytic activity. *FEBS Lett.* 509, 169–173

22. Tu, K., Li, J., Verma, V. K., Liu, C., Billadeau, D. D., Lampaech, G., Xiang, X., Guo, L., Dhanasekaran, R., Roberts, L. R., Shah, V. H., and Kang, N. (2015) Vasodilator-stimulated phosphoprotein promotes activation of hepatic stellate cells by regulating Rab11-dependent plasma membrane targeting of transforming growth factor β receptors. *Hepatology* 61, 361–374

23. de Assunção, T. M., Lombert, G., Cao, S., Yaqob, U., Mathison, A., Simonetti, D. A., Huebert, R. C., Urrutia, R. A., and Shah, V. H. (2014) New role for Kruppel-like factor 14 as a transcriptional activator involved in the generation of signaling lipids. *J. Biol. Chem.* 289, 15798–15809

24. Ancellin, N., Colmont, C., Su, J., Li, Q., Mittereder, N., Castermans, K., Simonetti, D. A., Huebert, R. C., Stefanski, M., Yang, L., Bi, Y., Beyder, A., Cao, S., Simari, R. D., Schatte, M., Yamane, D., Stoddard, M., Shah, V. H., and Rosen, H. R. (2015) Hepatitis C virus infection induces autocrine interferon signaling by human liver endothelial cells and release of exosomes, which inhibits viral replication. *Gastroenterology* 148, 392–402.e13

25. Luga, V., Zhang, L., Viloria-Petit, A. M., Ogumjimi, A. A., Iannou, M. R., Chiu, E., Buchanan, M., Hosein, A. N., Basik, M., and Wranza, J. L. (2012) Exosomes mediate stromal mobilization of autocrine Wnt-PCP signaling in breast cancer cell migration. *Cell* 151, 1542–1556

26. Valadi, H., Ekström, K., Bossios, A., Sjöstrand, M., Lee, J. J., and Lötvall, J. O. (2007) Exosome-mediated transfer of mRNAs and microRNAs is a novel mechanism of genetic exchange between cells. *Nat. Cell Biol.* 9, 654–659

27. Zhu, Q., Zou, L., Jagavelu, K., Simonetto, D. A., Huebert, R. C., Jiang, Z. D., DuPont, H., L., and Shah, V. H. (2012) Intestinal decontamination inhibits TLR4 dependent fibronectin-mediated cross-talk between stellate cells and endothelial cells in liver fibrosis in mice. *J. Hepatol.* 56, 893–899

28. Zou, L., Cao, S., Kang, N., Huebert, R. C., and Shah, V. H. (2012) Fibronectin induces endothelial cell migration through β1 integrin and Src-dependent phosphorylation of fibroblast growth factor receptor-1 at tyrosines 653/654 and 766. *J. Biol. Chem.* 287, 7190–7202

29. Yaqob, U., Cao, S., Shergill, U., Jagavelu, K., Geng, Z., Yin, M., de Assuncao, T. M., Cao, Y., Szabolics, A., Thorgeirsson, S., Schwartz, M., Yang, J. D., Ehman, R., Roberts, L., Mukhopadhyay, D., and Shah, V. H. (2012) Neuropilin-1 stimulates tumor growth by increasing fibronectin fibril assembly in the tumor microenvironment. *Cancer Res.* 72, 4047–4059

30. El-Sayed, A., and Harashima, H. (2013) Endocytosis of gene delivery vectors: from clathrin-dependent to lipid raft-mediated endocytosis. *Mol. Ther.* 21, 1118–1130

31. Singh, P., Carraher, C., and Schwarzjauer, B. E. (2010) Assembly of fibronectin extracellular matrix. *Annu. Rev. Cell Dev. Biol.* 26, 397–419

32. Marimpietri, D., Petretto, A., Raffaghello, L., Pezzolo, A., Gagliani, C., Tacchetti, C., Mauri, P., Melioli, G., and Pistoia, V. (2013) Proteome profiling of neuroblastoma-derived exosomes reveal the expression of proteins potentially involved in tumor progression. *PLoS ONE* 8, e75054

33. Higuchi, M., Onishi, K., Kikuchi, C., and Gotoh, Y. (2008) Scaffolding function of PAK in the PDK1-Akt pathway. *Nat. Cell Biol.* 10, 1356–1364

34. Konnek, M., Lynch, M., Mehta, S. H., Lai, M., Exley, M., Aldhal, N. H., and Schuppan, D. (2012) Circulating microparticles as disease-specific biomarkers of severity of inflammation in patients with hepatitis C or nonalcoholic steatohepatitis. *Gastroenterology* 143, 448–458

35. Konnek, M., Popov, Y., Libermann, T. A., Aldhal, N. H., and Schuppan, D. (2011) Human T cell microparticles circulate in blood of hepatitis patients and induce fibrolytic activation of hepatic stellate cells. *Hepatology* 53, 230–242

36. Melo, S. A., Luecke, L. B., Kahler, C., Fernandez, A. F., Gammon, S. T., Kaye, J., LeBlu, S. V., Mittendorf, E. A., Weitz, J., Rahbahi, N., Reissfelder, C., Pilarsky, C., Fragla, M. F., Piwnica-Worms, D., and Kalluri, R. (2015) Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature* 523, 177–182

37. Nojima, H., Freeman, C. M., Schuster, R. M., Japhtok, L., Schuster, R. M., Japtok, L., Kleuser, B., and Schuppan, D. (2015) Hepatocyte exosomes mediate liver repair and regeneration via sphingosine-1-phosphate. *J. Hepatol.* 10.1016/j.jhep.2015.07.030

38. Losee, C., Yui, D., Leung, L., Ingham, M., Kaler, M., Yao, X., Wu, W. W., Shen, R. F., Daniels, M. P., and Levine, S. J. (2009) Proteomic profiling of human plasma exosomes identifies PPARγ as an exosome-associated protein. *Biochem. Biophys. Res. Commun.* 378, 433–438

39. Caby, M. P., Lankar, D., Vincendeau-Scherrier, C., Raposo, G., and Bonnerot, C. (2005) Exosomal-like vesicles are present in human blood plasma. *Int. Immunol.* 17, 879–887

40. Henderson, N. C., and Sheppard, D. (2013) Integrin-mediated regulation of TGFβ in fibrosis. *Biochem. Biophys. Acta* 1832, 891–896

41. Damke, H., Binns, D. D., Ueda, H., Schmid, S. L., and Baba, T. (2001) Dynamin GTPase domain mutants block endocytic vesicle formation at the early stage of endocytosis. *J. Clin. Invest.* 113, 1138–1156

42. Yang, L., Popov, Y., Schuppan, D., Ordog, T., Mukhopadhyay, D., and Shah, V. (2012) Opposing roles of VEGF during liver fibrosis progression and resolution (Abstract). *Hepatology* 56, 768A

43. Giugliano, S., Kriss, M., Golden-Mason, L., Dobrinskikh, E., Stone, A. E., Soto-Gutierrez, A., Mitchell, A., Khetani, S. R., Yaman, J., Stoddard, M., Li, H., Shaw, G. M., Edwards, M. G., Lemon, S. M., Gale, M., Jr., Shah, V. H., and Rosen, H. R. (2015) Hepatitis C virus infection induces autocrine interferon signaling by human liver endothelial cells and release of exosomes, which inhibits viral replication. *Gastroenterology* 148, 392–402.e13
sine 1-phosphate receptor S1P2 triggers hepatic wound healing. FASEB J. 21, 2005–2013
52. Yang, L., Yue, S., Yang, L., Liu, X., Han, Z., Zhang, Y., and Li, L. (2013) Sphingosine kinase/sphingosine 1-phosphate (S1P)/S1P receptor axis is involved in liver fibrosis-associated angiogenesis. J. Hepatol. 59, 114–123
53. Kageyama, Y., Ikeda, H., Watanabe, N., Nagamine, M., Kusumoto, Y., Yashiro, M., Satoh, Y., Shimosawa, T., Shinozaki, K., Tomiya, T., Inoue, Y., Nishikawa, T., Ohtomo, N., Tanoue, Y., Yokota, H., Koyama, T., Ishimaru, K., Okamoto, Y., Takuwa, Y., Koike, K., and Yatomi, Y. (2012) Antagonism of sphingosine 1-phosphate receptor 2 causes a selective reduction of portal vein pressure in bile duct-ligated rodents. Hepatology 56, 1427–1438
54. Maceyka, M., Milstien, S., and Spiegel, S. (2009) Sphingosine-1-phosphate: the Swiss army knife of sphingolipid signaling. J. Lipid Res. 50, S272–276
55. Lee, H. D., Kim, Y. H., and Kim, D. S. (2014) Exosomes derived from human macrophages suppress endothelial cell migration by controlling integrin trafficking. Eur. J. Immunol. 44, 1156–1169
56. Conner, S. D., and Schmid, S. L. (2003) Regulated portals of entry into the cell. Nature 422, 37–44
57. Bobrie, A., Colombo, M., Raposo, G., and Théry, C. (2011) Exosome secretion: molecular mechanisms and roles in immune responses. Traffic 12, 1659–1668
58. Fitzner, D., Schnaars, M., van Rossum, D., Krishnamoorthy, G., Dibaj, P., Bakhti, M., Regen, T., Hansich, U. K., and Simons, M. (2011) Selective transfer of exosomes from oligodendrocytes to microglia by macropinocytosis. J. Cell Sci. 124, 447–458
59. Svensson, K. J., Christianson, H. C., Wittrup, A., Bourseau-Guilmain, E., Lindqvist, E., Svensson, L. M., Mörkelin, M., and Belting, M. (2013) Exosome uptake depends on ERK1/2-heat shock protein 27 signaling and lipid raft-mediated endocytosis negatively regulated by caveolin-1. J. Biol. Chem. 288, 17713–17724
60. Tian, T., Zhu, Y. L., Zhou, Y. Y., Liang, G. F., Wang, Y. Y., Hu, F. H., and Xiao, Z. D. (2014) Exosome uptake through clathrin-mediated endocytosis and macropinocytosis and mediating miR-21 delivery. J. Biol. Chem. 289, 22258–22267