Supporting Methods

Animal procedures and tissue specimens

Animal protocols were approved by the Institutional Animal Care and Use Committees of Nationwide Children's Hospital (Columbus, OH) or the University of Southern California (Los Angeles, CA). To induce hepatic fibrosis, male Swiss Webster mice (6–8 weeks) (n=9) received i.p. carbon tetrachloride (CCl_4; 175 µl in 1325 µl corn oil /kg; Sigma-Aldrich, St Louis, MO) three times per week for 5 weeks. Control mice received i.p. corn oil (1500 µl/kg) alone. Mice were sacrificed 36 hrs after the last injection for serum miR analysis or liver RNA analysis or histology after sequential in situ perfusion with PBS and 4% paraformaldehyde. In experiments to collect quiescent or activated HSC, male Swiss Webster mice (6–8 weeks) (n=9) received two i.p. injections, spaced 48 hrs apart, of, respectively, corn oil (1500 µl/kg) or CCl_4 (175 µl in 1325 µl corn oil /kg; Sigma-Aldrich) prior to harvesting HSC as described below. As an alternative method of inducing hepatic fibrosis, normal male FVB mice (6–8 weeks) (n=9) received i.p. thioacetic acid (TAA; 100mg/kg; Sigma-Aldrich) in saline three times per week for 4 weeks. Control mice received i.p. saline alone. Mice were sacrificed 72 hrs after the last injection and livers were harvested for histology or RNA analysis as described above. Additionally, hepatic histology or hepatic RNA was evaluated in male C57BL/6 mice (8 weeks) (n=3) that had been intragastrically fed ethanol and high fat diet (32% calories as corn oil) for 4 weeks to produce alcoholic steatohepatitis [1]. The ethanol dose was gradually increased to 32 g/kg/day to achieve blood alcohol levels of 250-350 mg/dL. Control mice were intragastrically fed isocaloric dextrose and high fat diet.

Histology

Perfused livers were fixed with 4% paraformaldehyde for 24 hrs and then embedded in paraffin. Sections of 5 µm thickness were cut and stained with hematoxylin and eosin (H & E). Collagen was detected by staining sections with 0.1% Sirius Red (Sigma-Aldrich).

Hepatic Stellate Cell (HSC) Culture

Primary HSC were isolated by in situ enzymatic dissociation and density gradient centrifugation from livers of oil- or CCl_4-injected mice, maintained in culture for 24 hrs in DMEM/F12 medium containing 10% fetal bovine serum (FBS), essentially as we have described previously [2], and processed for real-time polymerase chain reaction (RT-PCR) or *in situ* hybridization. Primary

HSC were also isolated from normal male Swiss Webster mice (6-8 weeks) using our established protocols [2] and spent medium from the cultured cells was replaced with fresh DMEM/F12/10%FBS medium on Day 1 and every other day as needed. HSC were split 1:3 every 5 days and used up to at passage 6 (P6). HSC identity and purity was verified by buoyant density, phase contrast microscopy, oil red staining, and immunostaining for αSMA, CCN2 or collagen α1. To detect lipid droplets, the cells were fixed in ice-cold 4% paraformaldehyde in PBS for 20 min at room temperature (RT) prior to incubation for 10 min in a saturated solution of oil red O (Polysciences, Warrington, PA) in isopropanol (Sigma-Aldrich). In some experiments, triplicate wells of Day 3 HSC were changed to low (1%) serum medium and incubated for up to 48 hrs in the presence of 0-100 mM ethanol. Human LX-2 HSC were cultured as described [2].

RNA extraction and RT-PCR

Total cellular RNA was extracted using a mirVana™ miRNA Isolation Kit (Ambion, Austin, TX, USA) and reverse transcribed using SuperScript II Reverse Transcriptase (Life Technologies, Carlsbad, CA, USA). Quantification of RNA levels for CCN2, miR-214 or collagen α1(I) (Supporting Table S2) was achieved by quantitative RT-PCR using an ABI PRISM 7000 Sequence Detection System and SYBR Green Master Mix (Life Technologies). Each reaction was run in triplicate and all samples were normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Negative controls were a non-reverse transcriptase reaction and a non-sample reaction. Serum microRNA was extracted using a miRNeasy Mini Kit (Qiagen) and reverse transcribed using a miscript II RT Kit (Qiagen). Quantification of RNA levels for miR-214 (Supporting Table S2) was achieved by quantitative RT-PCR using an ABI PRISM 7000 Sequence Detection System and SYBR Green Master Mix (Life Technologies). Each reaction was run in triplicate and all samples were normalized to small RNA U6.

Immunofluorescence

Fixed liver sections or primary passaged mouse HSC were incubated with NH1 anti-CCN2 IgY (5μg/ml)[3], anti-α-SMA (1:100, Dako Cytomatio, Denmark), anti-desmin (1:250, Abcam, Cambridge, MA) or anti-collagen α1 (1:250, Abcam, Cambridge, MA) followed by Alexa Fluor® 488 goat-anti chicken IgY, Alexa Fluor® 568 goat-anti rabbit IgG, and Alexa Fluor® 647 goat-anti mouse IgG (all at 1:1000; Life Technologies) for 1 hr at RT. The cells were mounted with Vectashield Mounting Medium containing 4',6-diamidino-2-phenylindole (DAPI) nuclear stain (Vector Laboratories, Burlingame, CA), and examined by confocal microscopy.

In-situ hybridization (ISH)

Fixed liver sections were hybridized with miR-214 or scramble sequence probes (Exiqon Inc., Woburn, MA) (Supporting Table S3) for 60 min at 55°C in 1x microRNA ISH Buffer (Exiqon Inc.) followed by washes with varying concentrations of SSC buffer at 55°C. Probes were detected with a monoclonal anti-digoxigenin-alkaline phosphatase antibody (1:800) (Roche, Indianapolis, IN) for 60 min followed by nitro-blue tetrazolium and 5-bromo-4-chloro-3'-indolyphosphate substrates (Roche) at 30°C for 2 hrs. Finally, slides were counterstained with Nuclear Fast Red™, mounted with Eukitt® Medium (VWR, Radnor, PA) and examined by confocal microscopy.

Fixed and permeabilized Day 1 mouse HSC were hybridized with probes to miR-214, CCN2, αSMA, or GFAP, or with a scrambled sequence (Exiqon Inc., Woburn, MA) (Supporting Table S3) for 30 mins at 58°C in 1x microRNA ISH Buffer (Exiqon Inc) followed by washes with varying concentrations of SSC buffer at 58°C. Cells were mounted in Prolong Gold Mounting Medium with DAPI (Life Technologies) and examined by confocal microscopy.

Characterization of exosomes in HSC conditioned medium

P6 primary mouse HSC, either control cells or those transfected with pre-mir-214 (100nM) by electroporation (Nucleofect Kit, Lonza), were incubated in fresh serum-free DMEM/F12 medium in T-75 culture flasks for 3hr or 36hr with 0-10 μM GW4869 exosome inhibitor. HSC conditioned medium (40ml) underwent serial centrifugation [4] to isolate exosomes which were then resuspended in 50-100 μl PBS. In some experiments, exosomes were mixed for 1 hour with fluorescent dye PKH26 (4mM; Sigma-Aldrich) prior to the first ultra-centrifugation step. Western blots were performed using anti-CD9 antibody (1:300; Lifespan Bioscience Inc., Seattle WA). Exosomal or cellular RNAs were isolated and the presence of miR-214 was determined by RT-PCR and normalized to total cellular GAPDH as described above. Similar procedures were used to characterize exosomes in conditioned medium of LX-2 cells cultured serum-free for 48 hrs.

Exosomes, isolated from the 48-hr conditioned medium of P6 mouse HSC that had been transfected with pre-mir-214 (100nM), were added for 24 hrs to cultures of P6 recipient mouse HSC to establish dose-dependent changes in expression of miR-214 or CCN2 mRNA by RT-PCR. Exosomes were also collected from 24-hr conditioned medium of primary cultures of HSC from Swiss Webster mice receiving two injections of oil or CCl_4. The presence of miR-214 was determined by RT-PCR and normalized to total cellular GAPDH. Western blots were performed

using anti-CD9 antibody (1:300; Lifespan Bioscience Inc., Seattle WA), or anti-flotillin antibody (1:500; Abcam) to verify comparable exosome recovery between the cultures.

Purified exosomes were analyzed by dynamic light scattering (632.8nm laser, 90^{o} detection angle) using a BI 200SM Research Goniometer System (Brookhaven Instruments Inc., Holtville NY). Zeta potential was determined with a ZetaPALS analyzer (Brookhaven Instruments Inc.). Exosomes were allowed to settle on carbon-coated 400-mesh copper grids (Electron Microscopy Sciences, Hatfield, PA), stained with 2% uranyl acetate, air-dried, and imaged by transmission electron microscopy (TEM) with a H-7650 microscope (Hitachi High Technologies America, Pleasanton, CA). Alternatively, purified exosomes were placed on carbon-coated grids which were then immediately blotted, plunge-frozen in liquid nitrogen-cooled ethane slush, and subjected to cryogenic TEM using a Tecnai G2 F20 microscope (FEI, Hillsboro, Oregon), essentially as described [5].

Co-culture system

Two-well silicone micro-culture devices (Ibidi Inc., Verona, WI, USA) were used to culture cells that were separated from each other by a central silicone wall (Supporting Figure S4). One well received donor cells comprising (i) Day 1 freshly isolated HSC; (ii) P6 activated HSC; or (iii) human LX-2 cells; these cells were either control or co-transfected by electroporation (Nulceofector, Lonza) with 2 µg green fluorescent protein (GFP)-CD9 and 2 µg pLemiR-214 and were cultured with 0-10 µg/ml GW4869. After 12 hrs, the other well was seeded with recipient cells that comprised (i) P6 activated HSC, (ii) LX-2 cells; or (iii) HepG2 hepatocytes, each of which had been transfected with parental miR-Selection Fire-Ctx lentivector or the same vector containing either wild type or mutant CCN2 3'-UTR. After 12 hrs, the silicone wall separating donor cells from recipient cells was excised to allow direct communication between the cells for the following 24hrs. Control experiments included exposure of recipient HSC to cell-free donor micro-wells containing pre-mir-214 in the medium at the same concentration as used for donor HSC transfection. Donor or recipient cells were imaged for fluorescence, and luciferase activity was measured in triplicate using the Dual Luciferase Reporter Assay System. Firefly luciferase activity in pre-mir-214 transfected cells was compared to that in non-transfected cells, with *Renilla* luciferase activity used for normalization.

Hepatocyte Cell Isolation

Mouse hepatocytes were isolated from 6-10 week old male Swiss Webster mice using slight modifications of published procedures [6]. Briefly, livers were perfused via the portal vein with Krebs-Ringer Hepes buffer (KRB) containing 1.7 mM EDTA followed by KRB containing 8 M $CaCl_2$ and collagenase IV (0.7 mg/ml) (Sigma). The liver was then removed and dispersed via shaking and alternately rinsing with KRB. The sample was passed sequentially through 120 µm and 70 µm pore mesh filters and the resulting cell suspension was centrifuged at 40 x *g* for 5 minutes. Cells were resuspended in Percoll solution and KRB (1:1) and centrifuged at 500 x *g* for 5 minutes. The pellet was collected and washed in Hepatocyte Culture Medium (Lonza) at 40 x *g* for 5 minutes. Cells were then placed in a 24-well plate (1×10^6 cells/well) for up to 4 hours in the presence of mouse P6 HSC-derived exosomes that had been pre-labeled with PKH26 or isolated from HSC transfected with RFP-pre-mir-214 (4 µg). Cell aliquots were subsequently washed in PBS, cytospun for 6 min at 1000 rpm, immediately mounted using ProLong Gold Antifade mounting medium with DAPI (Life Technologies), and examined by confocal microscopy.

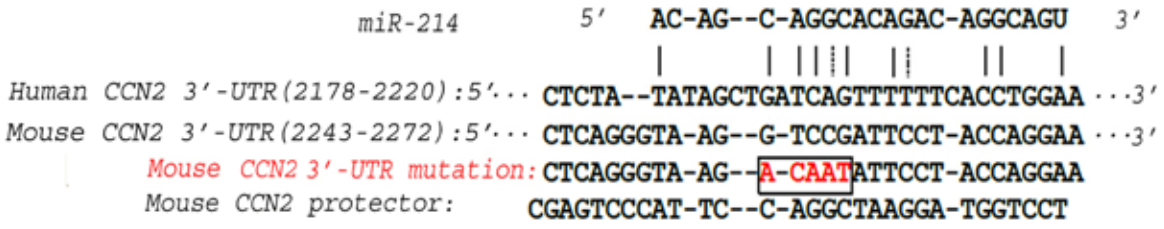
Statistical Analysis

All experiments were performed at least three times with triplicate measurements, with data expressed as mean ± s.e.m. The data from RT-PCR, viability assays, or luciferase activity assays were analyzed by student's *t*-test using Sigma plot 11.0 software (SPSS Inc., Chicago, IL) and *P* values < 0.05 were considered statistically significant.

Supporting References

1. Tsukamoto H, Mkrtchyan H, Dynnyk A. Intragastric ethanol infusion model in rodents. Methods Mol Biol 2008;447:33-48.
2. Chen L, Charrier AL, Leask A, French SW, Brigstock DR. Ethanol-stimulated differentiated functions of human or mouse hepatic stellate cells are mediated by connective tissue growth factor. J Hepatol 2011;55:399-406.
3. Charrier AL, Brigstock DR. Connective tissue growth factor production by activated pancreatic stellate cells in mouse alcoholic chronic pancreatitis. Lab Invest 2010;90:1179-1188.
4. Thery C, Amigorena S, Raposo G, Clayton A. Isolation and characterization of exosomes from cell culture supernatants and biological fluids. Curr Protoc Cell Biol 2006;Chapter 3:Unit 3 22.
5. Fang PA, Margolis HC, Conway JF, Simmer JP, Dickinson GH, Beniash E. Cryogenic transmission electron microscopy study of amelogenin self-assembly at different pH. Cells Tissues Organs 2011;194:166-170.
6. Shulman M, Nahmias Y. Long-term culture and coculture of primary rat and human hepatocytes. Methods Mol Biol 2013;945:287-302.

A



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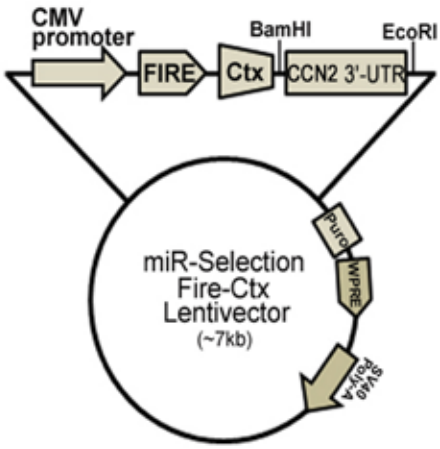
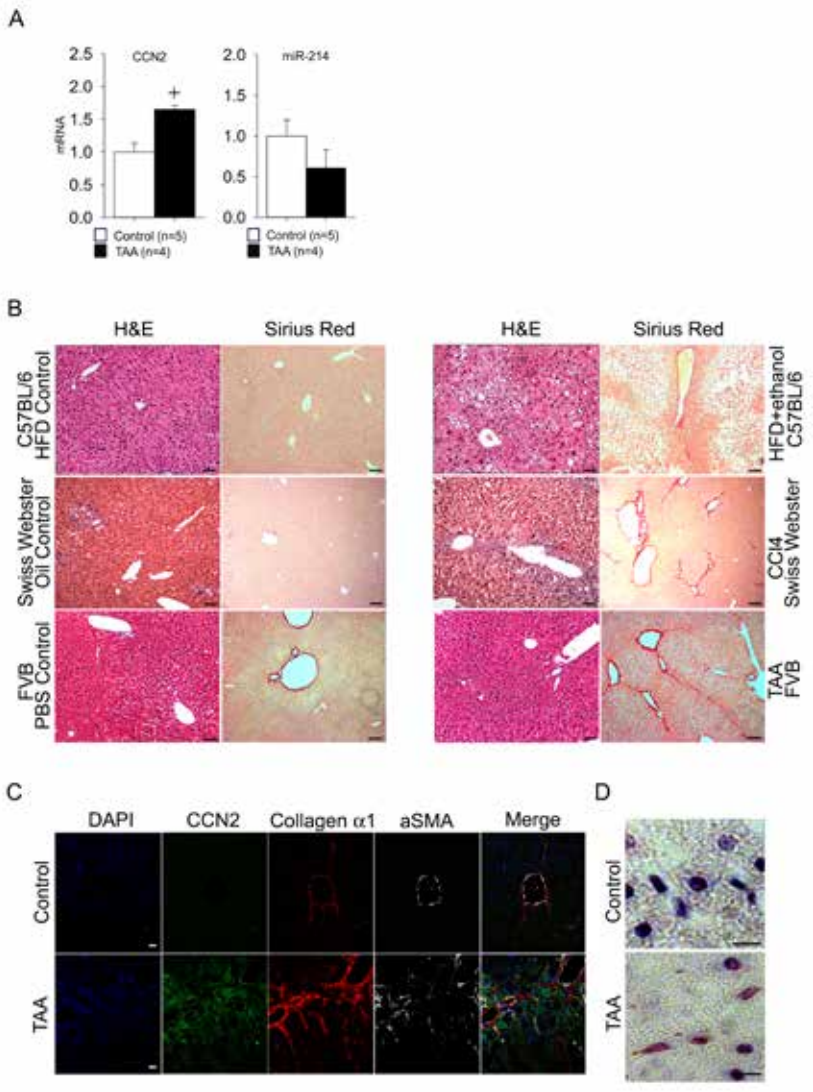
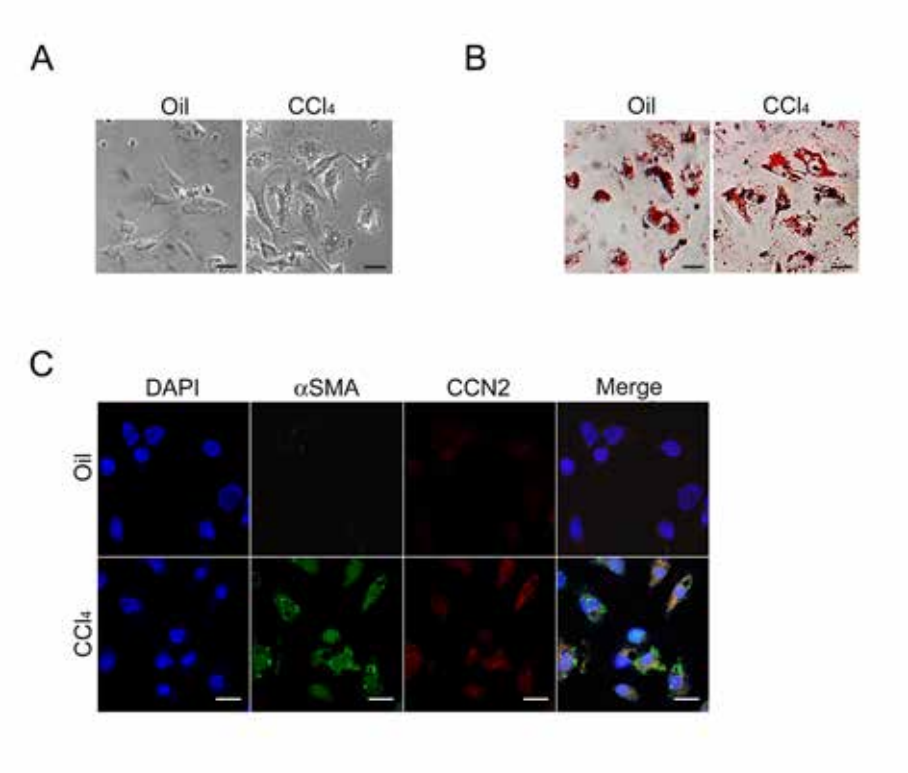


Supporting Fig. S1. *(A)* Sequence alignment of miR-214 with its binding site in the 3'-UTR of human (Genbank SEQ ID: BC087839.1) or mouse (Genbank SEQ ID: BC006783.1) CCN2. The 3'-UTR protector sequence and the region in the 3'-UTR selected for substitution mutagenesis are shown. *(B)* Organization of the miR-Selection Fire-Ctx lentivector containing CCN2 3'-UTR.

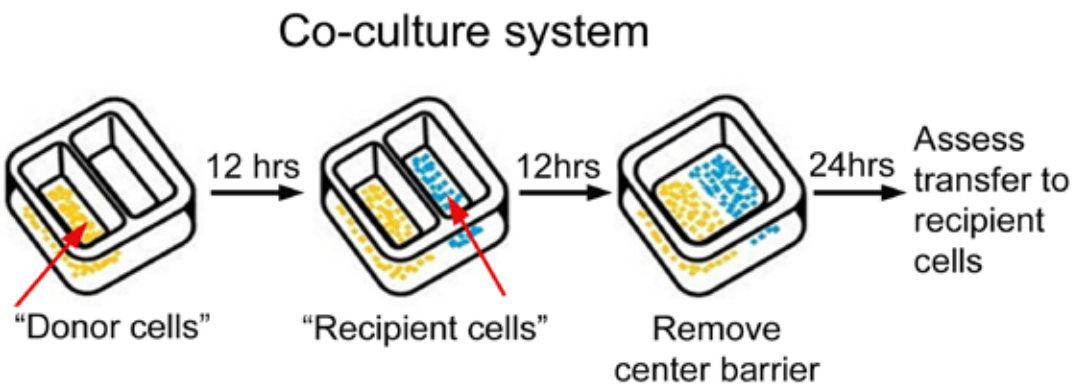


Supporting Fig. S2. *(A)* Hepatic expression of CCN2 mRNA or miR-214 assessed by RT-PCR and normalized to GAPDH mRNA after 4-week administration of TAA in FVB mice. *(B)* H & E staining or Sirius Red staining in control mice versus those receiving chronic administration of ethanol, CCl_4 or TAA. *Scale bar:* 50μm. *(C)* Immunohistochemical detection of CCN2, αSMA, or collagen α1 in livers of control or TAA-treated mice. *(D)* ISH for miR-214 in the livers of control mice or in the fibrotic regions of TAA-treated mice. *Scale bar:* 20 μm.



Supporting Fig. S3. *(A)* Phase contrast microscopy, *(B)* oil red staining, or *(C)* immunostaining for CCN2 or αSMA in HSC cultured for one day after isolating the cells 36 hrs after the last of two oil or CCl_4 injections spaced 48 hrs apart. *Scale bar:* 20μm

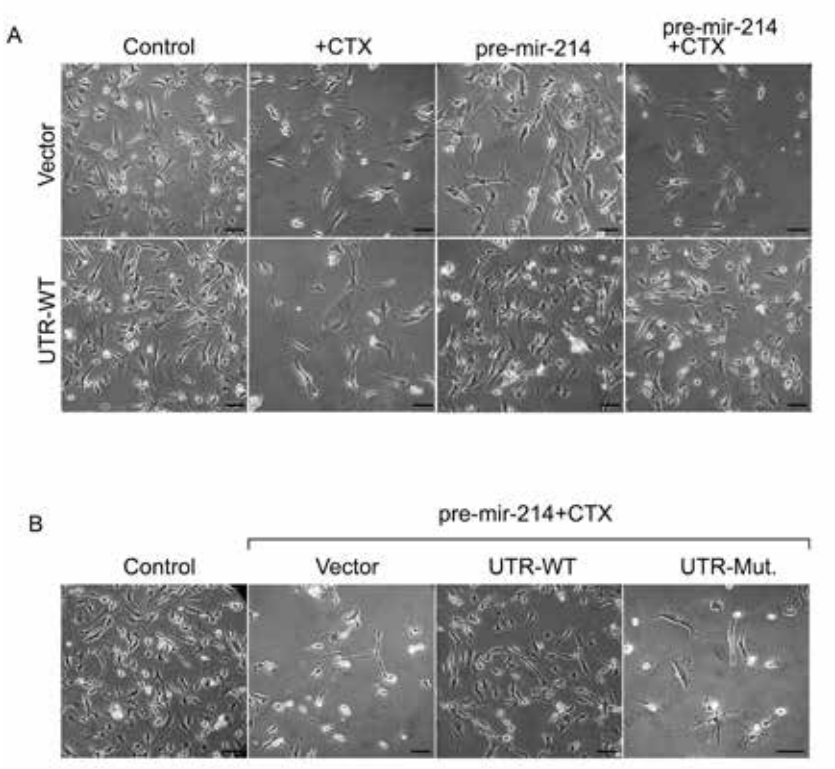
A



B

Donor cells	Recipient cells	Figure
P6 mouse HSC +CD9-GFP/miR-214-RFP ± GW4869	P6 mouse HSC (no transfection)	Figure 5A
P6 mouse HSC +pre-mir-214 ± GW4869	P6 mouse HSC + CCN2 3' UTR-WT or UTR-mutant	Figure 5B
D1 or P6 mouse HSC ± GW4869	P6 mouse HSC + Vector or CCN2 3' UTR-WT or UTR Mutant	Figure 5C
P6 mouse HSC +pre-mir-214 ± GW4869	P6 mouse HSC	Figure 5D
LX-2 cells +pre-mir-214 ± GW4869	LX-2 cells + Vector or CCN2 3' UTR-WT or UTR Mutant	Figure 6D
LX-2 cells +pre-mir-214 ± GW4869	HepG2 cells + CCN2 3' UTR	Figure 6E

Supporting Fig. S4. *(A)* Co-culture system comprising silicone micro-culture devices (outer-dimensions: 8.4 x 8.4 x 5 mm) containing two wells (0.22cm^2 growth area per well) separated by a shared silicone wall, removal of which results in a cell-free gap of 500 ± 50 µm. Approximately 3000 control or GFP-CD9- and RFP-miR-214-co-transfected donor cells were placed in one well in 70 µl medium for 12 hrs, with or without GW4869. Next, ~3000 recipient cells transfected with wild type or mutant CCN2 3'-UTR miR-Selection Fire-Ctx lentivector or the parental vector control were placed in the other well in 70 µl medium for 12 hrs. The central divider was then removed and the cells were co-cultured for a further 24 hrs prior to analysis by fluorescence microscopy or luciferase assay. *(B)* Summary of co-culture conditions used in the studies described.



Supporting Fig. S5. *(A)* P6 mouse HSC were transfected with parental Fire-Ctx plasmid ("vector") or Fire-Ctx plasmid containing wild-type CCN2 3'-UTR ("UTR-WT") with or without co-transfection with pre-mir-214. Some cell cultures were also treated with CTX, as indicated. Photomicrographs of the cells after 4 days in culture are shown. *(B)* Cells were treated as in *(A)* except that some cultures were transfected with Fire-Ctx plasmid containing a mutated CCN2 3'-UTR ("UTR-Mut."). *Scale bars*: 100µm.

Supporting Table S1. Altered expression of selected genes by miR-214 in activated mouse HSC.

Gene Name	Fold Regulation
Interleukin 10 (IL-10)	-7.4
Interleukin 1 alpha (IL-1α)	-12.9
Integrin alpha 3 ($Itg\alpha_3$)	-461.8
Integrin beta 8 ($Itg\beta_8$)	-87.8
Matrix metalloproteinase 13 (MMP13)	-285.4
Matrix metalloproteinase 2 (MMP2)	-145.5
Matrix metalloproteinase 8 (MMP8)	-16.2
Platelet derived growth factor, alpha (PDGFα)	-190.1
Tissue inhibitor of metalloproteinase 1 (TIMP1)	-29.8

RNA expression in P6 mouse HSC transfected for 24 hours with pre-mir-214 versus control (see Materials and Methods) was assessed using a Mouse Fibrosis RT^2 Profiler PCR Array (Qiagen).

Supporting Table S2. Primers used for RT-PCR

Gene	GenBank accession number	Primers		Product size (bp)
		Sense	Anti-sense	
CTGF (mouse)	NM_010217	5' CACTCTGCCAGTGGAGTTCA 3'	5' AAGATGTCATTGTCCCCAGG 3'	111
miR-214 (mouse)	NR_029796	5' ACAGCAGGCACAGACAGGCA 3'	Universal anti-sense	20
Collagen α1(I) (mouse)	NM_007742	5' GCCCGAACCCCAAGGAAAAGAAGC 3'	5' CTGGGAGGCCTCGGTGGACATTAG 3'	148
CTGF (human)	NM_001901	5' AATGCTGCGAGGAGTGGGT 3'	5' CGGCTCTAATCATAGTTGGGTCT 3'	94
GAPDH (mouse)	NM_002046	5' TGCACCACCAACTGCTTAGC 3'	5' GGCATGGACTGTGGTCATGAG 3'	66

Supporting Table S3. Probes used for *in situ* hybridization

Gene/probe	accession number	Sequence
CTGF (mouse)	NM_010217	5' ACTCAGTTCAAGTTATAGTCT Bio 3'
GFAP (mouse)	NM_010277	5' TATCTAAGGGAGAGCTGGAGCA Bio 3'
miR-214 (mouse)	NR_029796	5' 56-FAM ACTGCCTGTCTGTGCCTGCTG 3'
miR-214 (mouse)	NR_029796	5' DigN ACTGCCTGTCTGTGCCTGCTGT DigN 3'
αSMA (mouse)	NM_007392	5' AAAGGAACTGGAGGCGCTGAT Bio 3'
scramble		5' 56-FAM GTGTAACACGTCTATACGCCCA 3'