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Supplemental Information

Exosomal Transmission of MicroRNA from HCV Replicating Cells Stimulates Transdifferentiation in Hepatic Stellate Cells

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Supplementary Figures

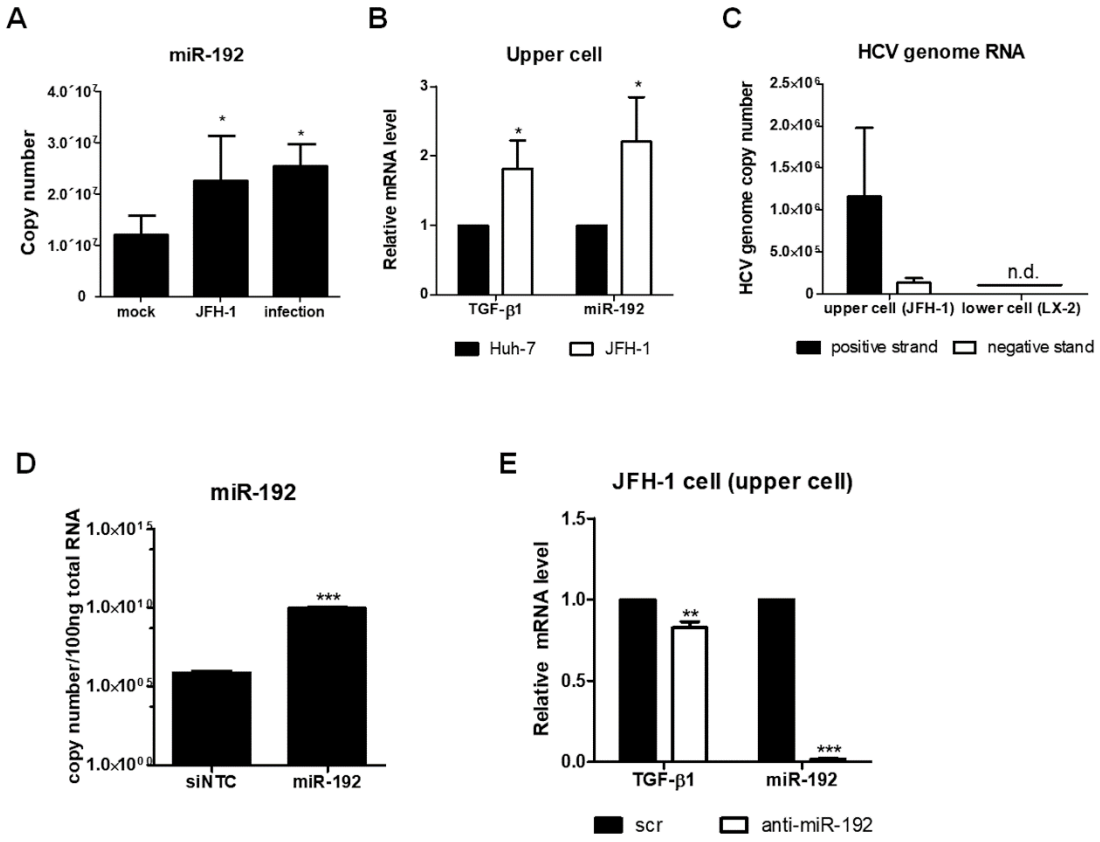

Figure S1. RNA levels in co-cultured upper cells (Huh-7 or HCV replicating cells) and HSCs. (A) Copy numbers of miR-192 per 100 ng of total RNA in naïve Huh-7 cells (mock, $1.20 \pm 1.00 \times 10^6$ copies), JFH-1 stable cells (JFH-1, $2.26 \pm 1.96 \times 10^7$ copies), and JFH-1-infected cells (infection, $2.54 \pm 0.85 \times 10^7$ copies). (B) Levels of TGF-β1 mRNA and miR-192 in naïve Huh-7 or JFH-1 stable cells co-cultured with LX-2 cells. (C) JFH-1 genome RNA levels in co-cultured JFH-1 stable cells and LX-2 cells. Copy numbers of HCV genome RNA per 1 μg of total RNA were assessed using qRT-PCR and calculated using a standard curve. The copy numbers of the positive and negative strands were $1.16 \pm 2.16 \times 10^6$ and $1.34 \pm 1.40 \times 10^5$, respectively, in JFH-1 stable cells. (D) Levels of miR-192 in LX-2 cells, which were transfected with siNTC or miR-192 mimic RNA. (E) The expression of TGF-β1 mRNA and miR-192 in JFH-1 stable cells, which were transfected with scramble siRNA (scr) or anti-miR-192 RNA. RNA levels were normalized to those of 18S or GAPDH mRNA in each sample. All data are presented as the means of at least three independent replicates, each performed in triplicate. Error bars represent SEM, and n.d. indicates not determined. *P* values were determined using a one-tailed unpaired Student's *t*-test. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

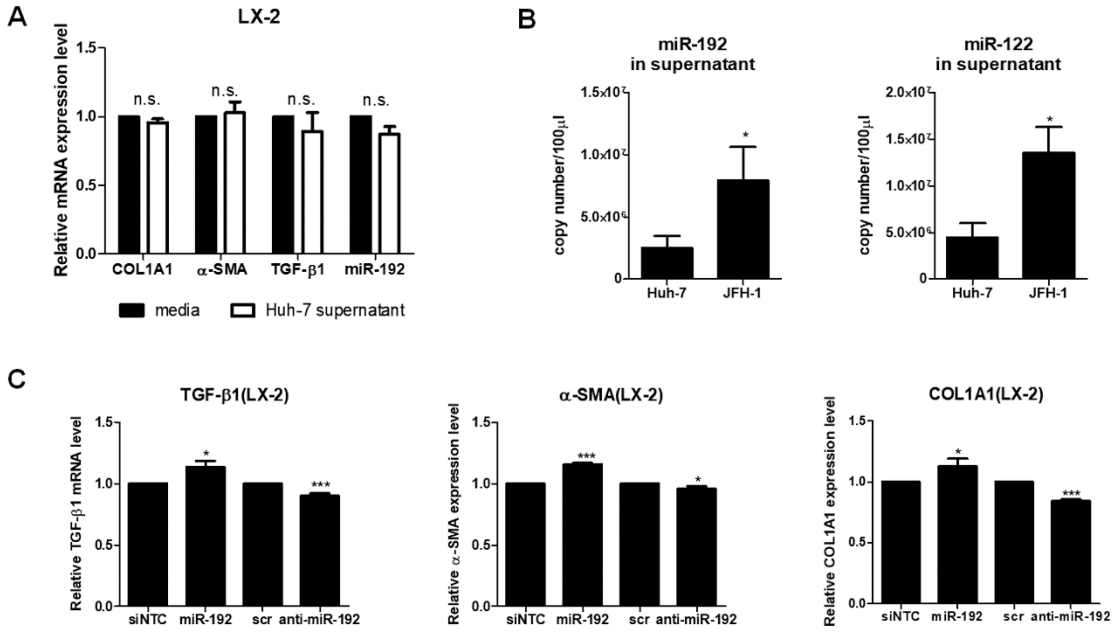

Figure S2. Effects of cell-free supernatant on HSCs. (A) The RNA levels of COL1A1, α-SMA, TGF-β1, and miR-192 in LX-2 cells exposed to supernatant from naïve Huh-7 cells were quantified relative to those in cells treated with control media. (B) Quantification of miR-192 (*left panel*) and miR-122 levels (*right panel*) in naïve Huh-7 cell- or JFH-1 stable cell-free supernatant using qRT-PCR and standard curves (miR-192, $2.51 \pm 2.34 \times 10^6$ copies per 100 μl of Huh-7 supernatant and $7.92 \pm 6.65 \times 10^6$ copies per 100 μl of JFH-1 supernatant; miR-122, $4.49 \pm 3.36 \times 10^6$ copies per 100 μl of Huh-7 supernatant and $1.35 \pm 0.62 \times 10^7$ copies per 100 μl of JFH-1 supernatant). Results are expressed as the means of at least three independent experiments, each performed in triplicate. (C) miR-192 mimic RNA or anti-miR-192 was transfected into Huh-7 cells for 48 h, and then supernatants from the cells were used to treat LX-2 cells for 72 h. The mRNA expression levels of the intracellular fibrogenic makers COL1A1 and α-SMA, as well as those of TGF-β1, in LX-2 cells were analyzed by qRT-PCR and shown relative to those in cells treated with supernatant from cells transfected with negative-control miRNA (siNTC) or scramble (scr) RNA. RNA levels were normalized to GAPDH mRNA for each sample, and the results are expressed as means of at least three independent experiments, each performed in triplicate. Error bars represent SEM. *P* values were determined using a one-tailed unpaired . $*p < 0.05$, $***p < 0.001$, n.s., non-significant.

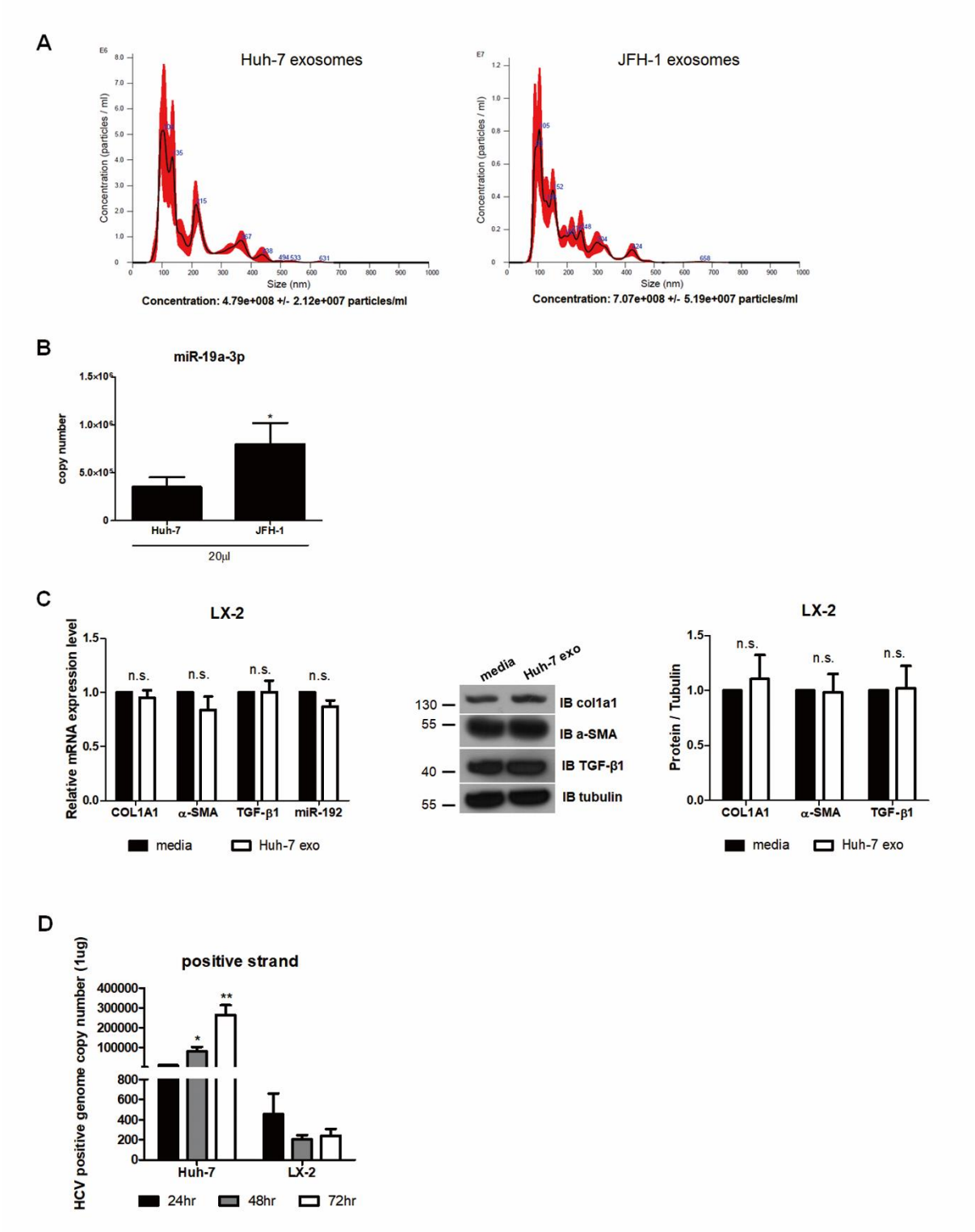

Figure S3. Analysis of exosomes from naïve Huh-7 and HCV replicating cells. (A) NanoSight analysis of particle numbers of exosomes from the supernatant of naïve Huh-7 or JFH-1 stable cells. The size of the purified exosomes was about 100 nm-120 nm. Huh-7 exosome concentration was $4.79 \pm 0.21 \times 10^8$ and JFH-1 exosome concentration was $7.07 \pm 0.51 \times 10^8$. (B) Quantification of miR-19-3p levels within exosomes isolated from naïve Huh-7 or JFH-1 stable cells by qRT-PCR. The miR-19-3p copy number is shown as the mean of three independent experiments, each performed in triplicate ($3.37 \pm 2.27 \times 10^5$ copies per 20 μl of Huh-7 exosomes and $7.99 \pm 4.37 \times 10^5$ copies per 20 μl of JFH-1 exosomes). (C) The RNA levels of COL1A1, α-SMA, TGF-β1, and miR-192 in

LX-2 cells exposed to exosomes from naïve Huh-7 cells free supernatant (Huh-7 exo) were quantified relative to those in cells treated with control media (*left panel*). Immunoblot analysis of the fibrosis markers COL1A1 and α-SMA, as well as TGF-β1, in LX-2 cells treated with Huh-7 exo or media (*middle panel*) and quantification of their levels relative to those of tubulin (*right panel*). (D) Copy numbers of HCV genome RNA in Huh-7 cells or LX-2 cells after treatment with exosome from JFH-1 stable cells were assessed using qRT-PCR. Error bars represent the SEM. *P* values were determined using a one-tailed unpaired Student's *t*-test (*$p < 0.05$, **$p < 0.01$, n.s., non-significant.).

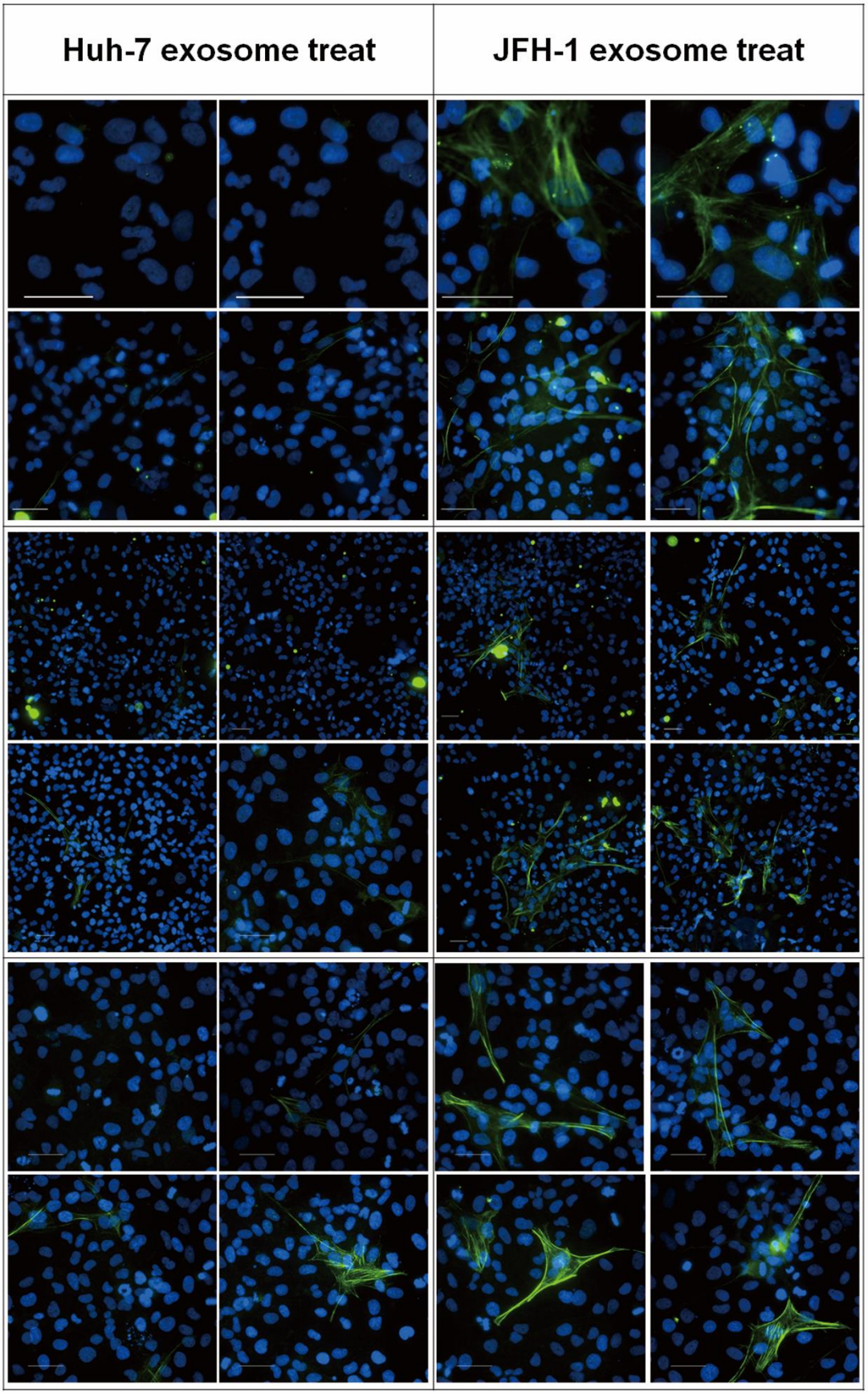
Huh-7 exosome treat
JFH-1 exosome treat

Figure S4. Effect of exosomes on HSC activation.

LX-2 cells were treated with Huh-7 or JFH-1 exosomes. The representative fluorescence images of α-SMA (Alexa Fluor 488, green) and DAPI (nuclei, blue) staining in cells measured in more than five different fields from at least three independent experiments are presented. The scale bar represents 50 μm.

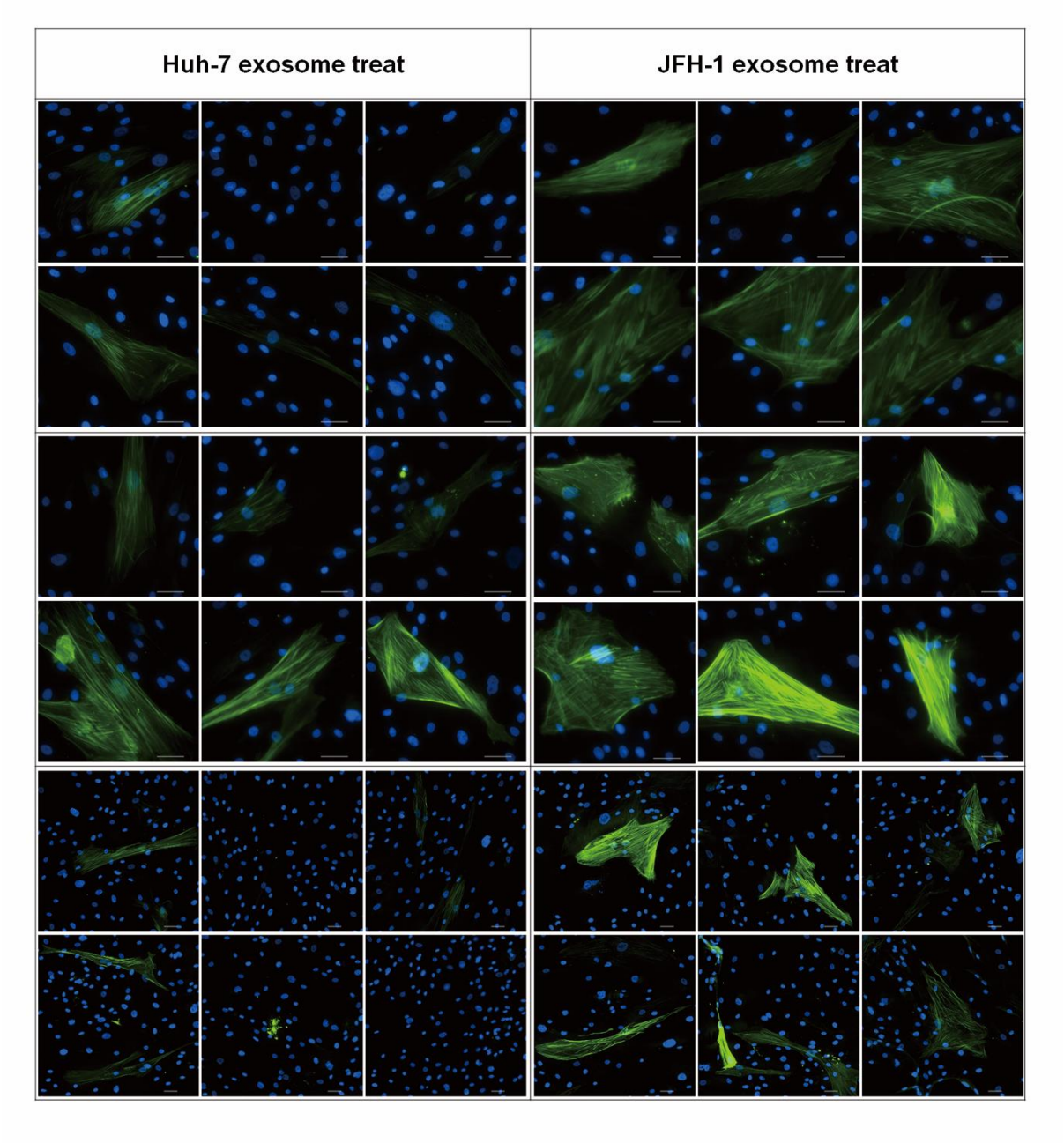

Figure S5. Effect of exosomes on primary HSCs activation.

Primary HSC cells were treated with Huh-7 or JFH-1 exosomes. The representative fluorescence images of α-SMA (Alexa Fluor 488, green) and DAPI (nuclei, blue) staining in cells measured in more than five different fields from at least three independent experiments are presented. The scale bar represents 50 μm.

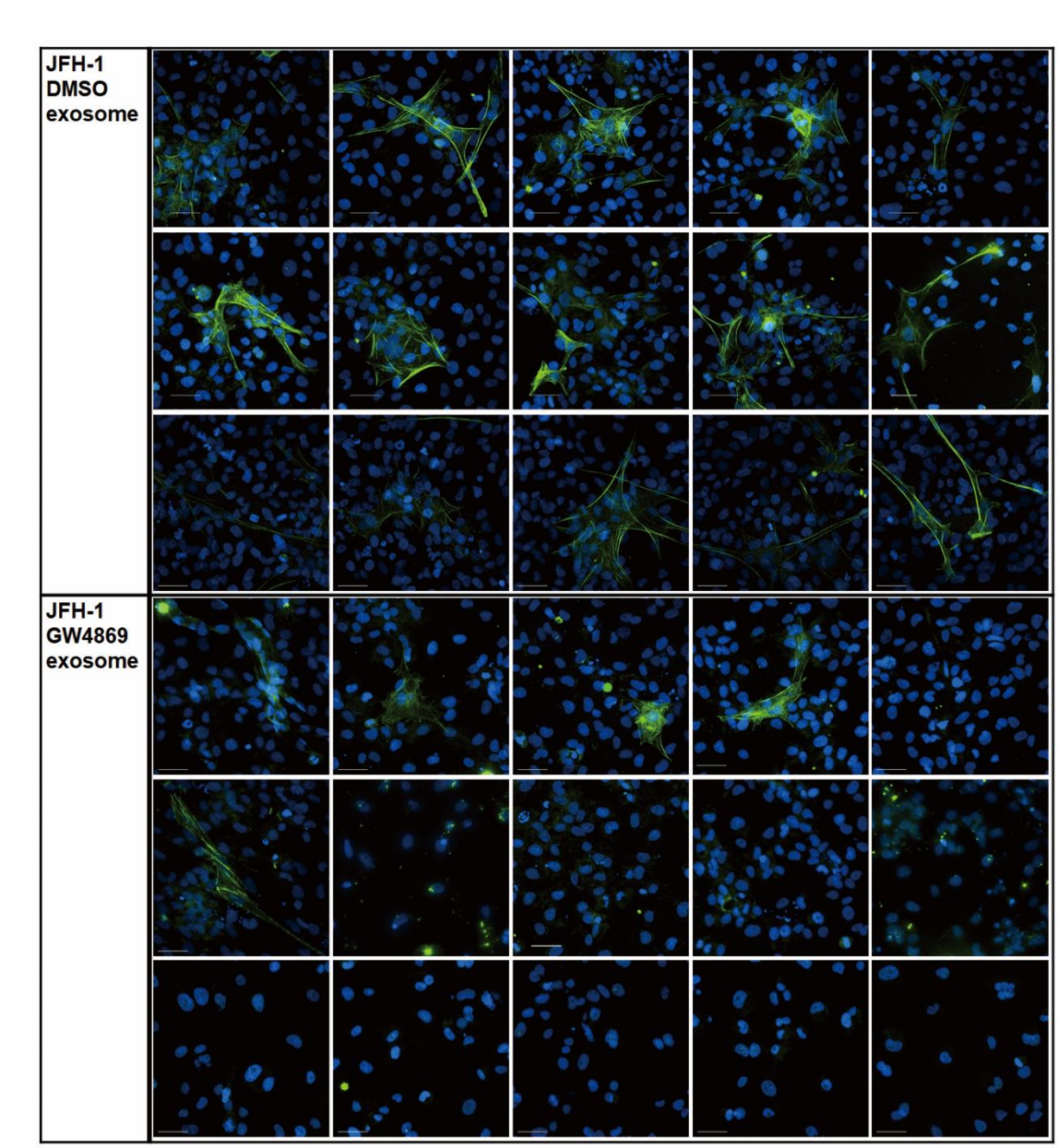

Figure S6. Effects of exosomes from DMSO- or GW4869-treated HCV replicating hepatocytes. Fluorescence intensity of α-SMA protein in LX-2 cells treated with DMSO or GW4869 exosomes. Representative images of α-SMA (Alexa Fluor 488, green) and DAPI (nuclei, blue) staining in cells in more than five fields per group from at least three independent experiments are shown. The scale bar represents 50 μm.

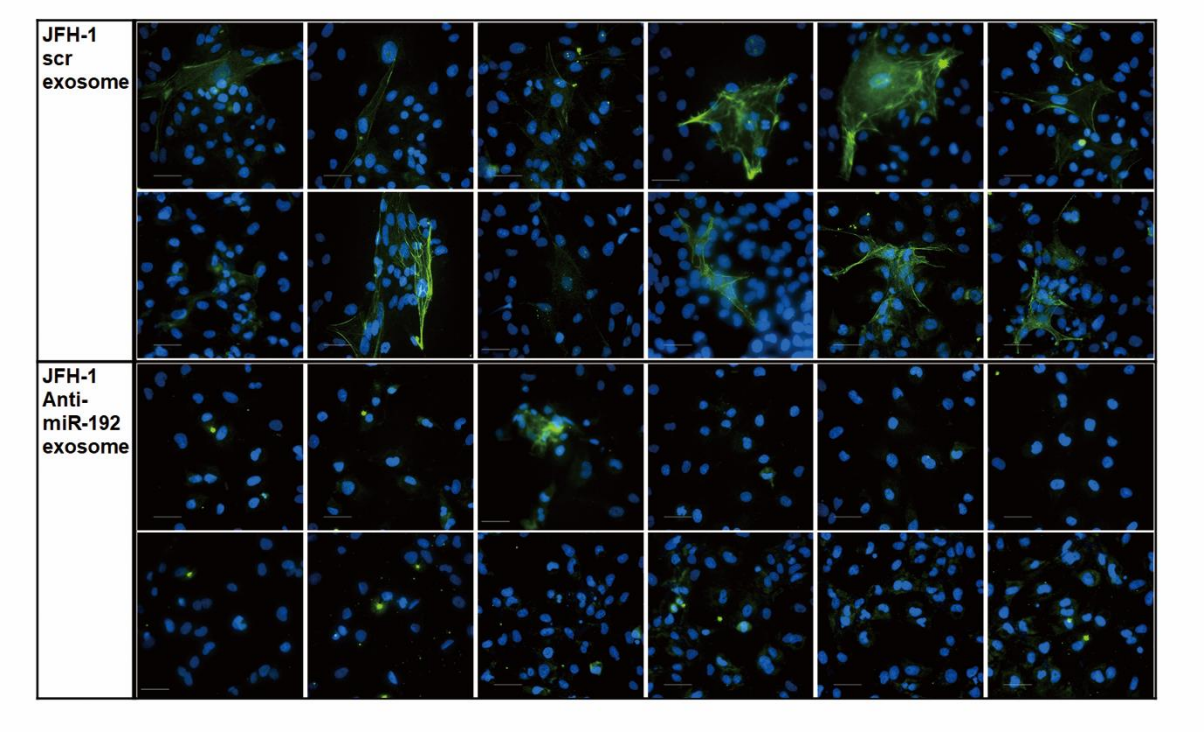

Figure S7. Effects of exosomes from scramble (scr)- or anti-mir-192–transfected HCV replicating hepatocytes. Fluorescence intensity of α-SMA measured in LX-2 cells treated with scr or anti-miR-192 exosomes. Representative images of α-SMA (Alex Fluor 488, green) and DAPI (nuclei, blue) staining in cells in more than five fields per group from at least three independent experiments are shown. The scale bar represents 50 μm.

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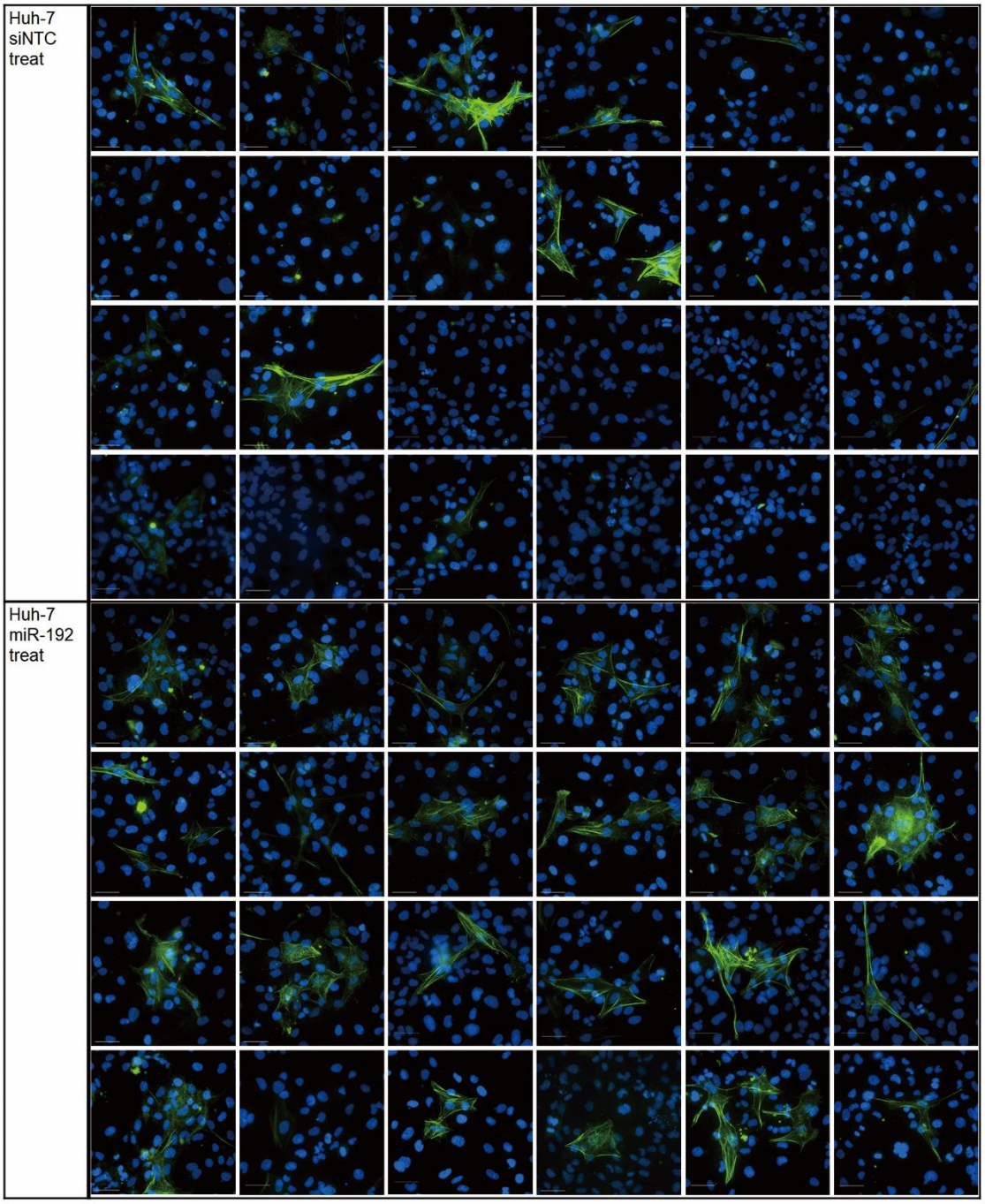

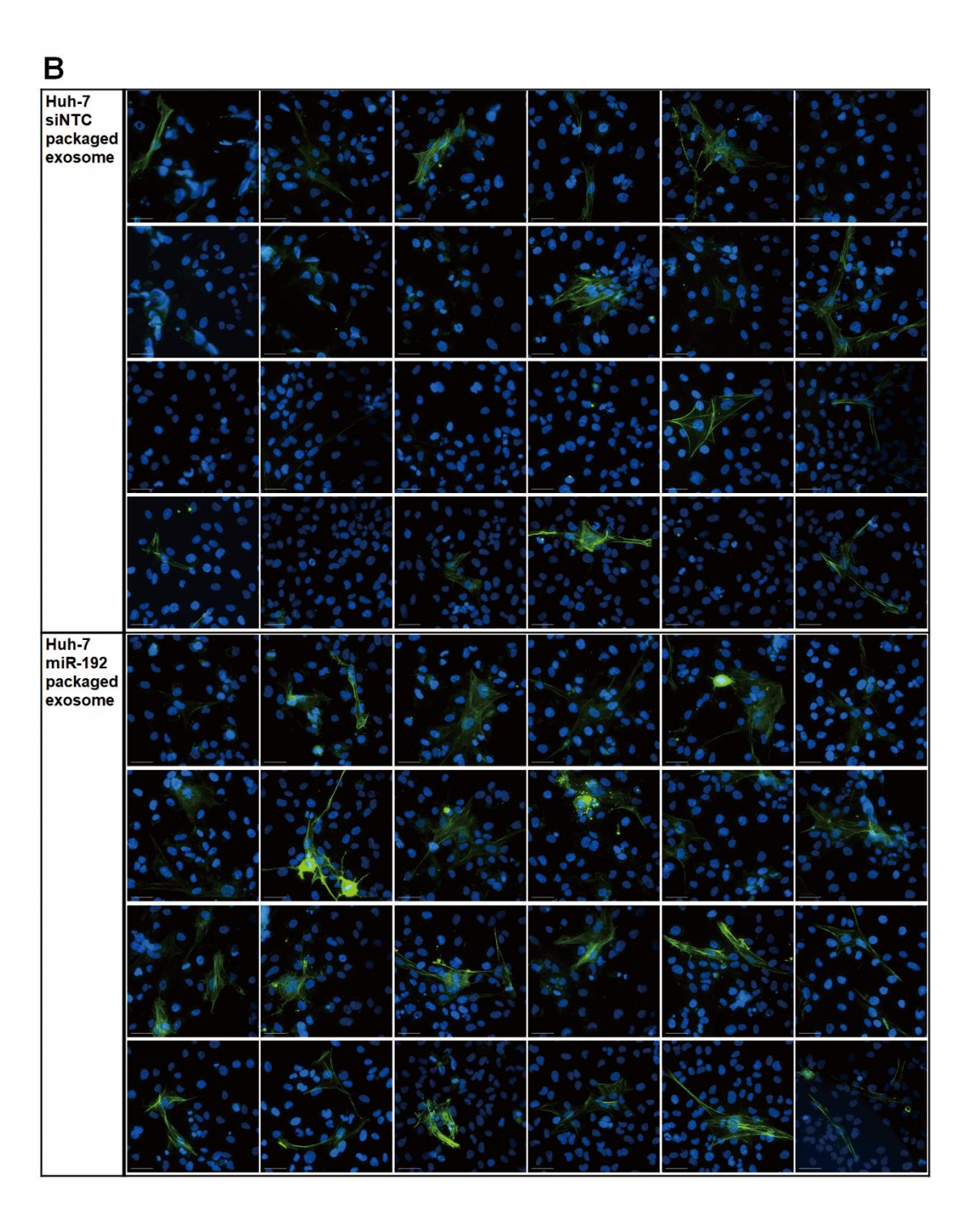

Figure S8. Effects of exosomes from miR-192-transfected hepatocytes or exosomes packaged with miR-192. (A) Fluorescence intensity of α-SMA protein in LX-2 cells treated with siNTC or miR-192 exosomes. (B) Fluorescence intensity of α-SMA protein in LX-2 cells treated with siNTC- or miR-192-packaged exosomes. Representative images of α-SMA (Alexa Fluor 488, green) and DAPI (nuclei, blue) staining in cells among more than five fields per group from at least three independent experiments are shown. The scale bar represents 50 μm.

Supplementary Tables

Table S1. RNA sequences for transfection

name	Sequence (5’ to 3’)
miR-192 mimic RNA sense	5′-CUGACCUAUGAAUUGACAGCC-3′
miR-192 mimic RNA anti-sense	5′-CUGCCAAUUCCAUAGGUCACAG-3′
siNTC RNA sense	5′-UUCUCCGAACGUGUCACGUTT-3′
siNTC RNA anti-sense	5′-ACGUGACACGUUCGGAGAATT-3′
Anti-miR-192 RNA	5′-GGCUGUCAAUUCAUAGGUCAG-3′
Anti-scramble RNA	5′-CAUUAAUGUCGGACAACUCAAU-3′

Table S2. Oligonucleotides sequences of primers for real-time PCR

Primer name	Sequence (5' to 3')
JFH-1 forward (F)	5′-CGACCAGTACCACCATCCTT-3′
JFH-1 reverse (R)	5′-AGCACCTTACCCAGGCCT-3′
18S rRNA F	5′-GTA ACCCGTTGA ACCCCA TT-3′
18S rRNA R	5′-CCATCCAATCGGTAGTAGCG-3′
TGF-β1 F	5′-CAAGGATCTGGGCTGGAAGTGGA-3′
TGF-β1 R	5′-CCAGGACCTTGCTGTACTGCGTGT-3′
COL1A1 F	5′-CGGTGTGACTCGTGCAGC-3′
COL1A1 R	5′-ACAGCCGCTTCACCTACAGC-3′
α-SMA F	5′-CCGACCGAATGCAGAAGGA-3′
α-SMA R	5′-CAGAGTATTTGCGCTCCGAA-3′
GAPDH F	5'-TGACATCAAGAAGGTGGTGA-3'
GAPDH R	5'- TCCACCACCCTGTTGCTGTA-3'

Abbreviations: F, forward; R, reverse