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Supplemental Information

Generation of functionally competent hepatic stellate cells from human stem cells to model liver fibrosis *in vitro*

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Supplementary figures and Legends

Figure S1

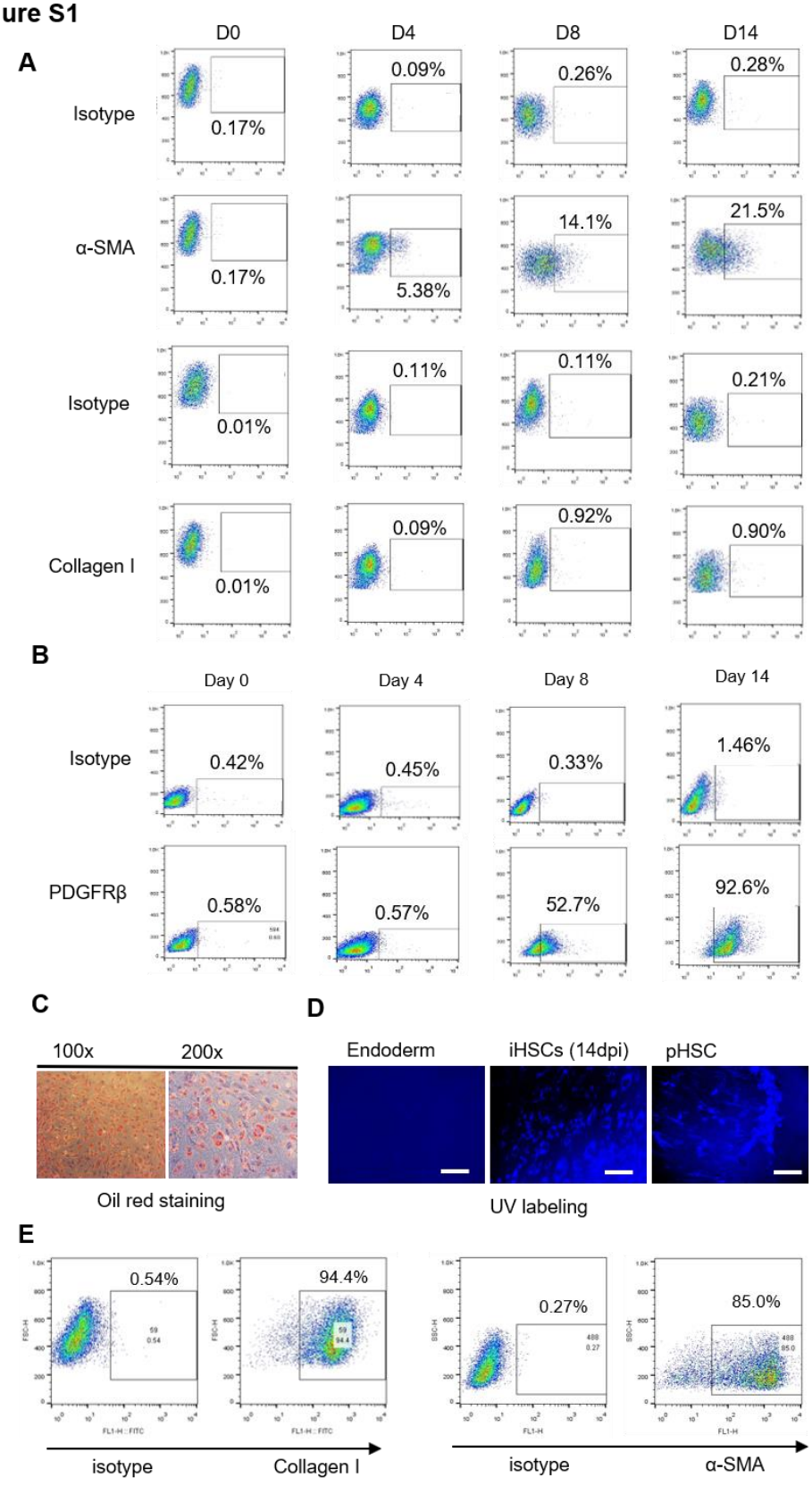

Figure S1. Characterization of endoderm derived iHSCs from hESCs (H9).

Related to Figure 1. (A) Representative flow cytometry plot of α-SMA and collagen I at day 0, 4, 8 and 14 differentiation from hESCs to iHSCs. Positive gates were defined based on the isotype control. (B) Flow cytometry analysis of PDGFRβ at day 0, 4, 8, 14 during the iHSCs differentiation. (C) Representative images of oil red staining analysis of the iHSCs. (D) UV beaching of endoderm, iHSC and pHSC under microscope. Scale bars, 100 μm. (E) Flow cytometry analysis of α-SMA and collagen I in iHSCs during the activation by TGFβ. Positive gates were defined based on the isotype control.

Figure S2

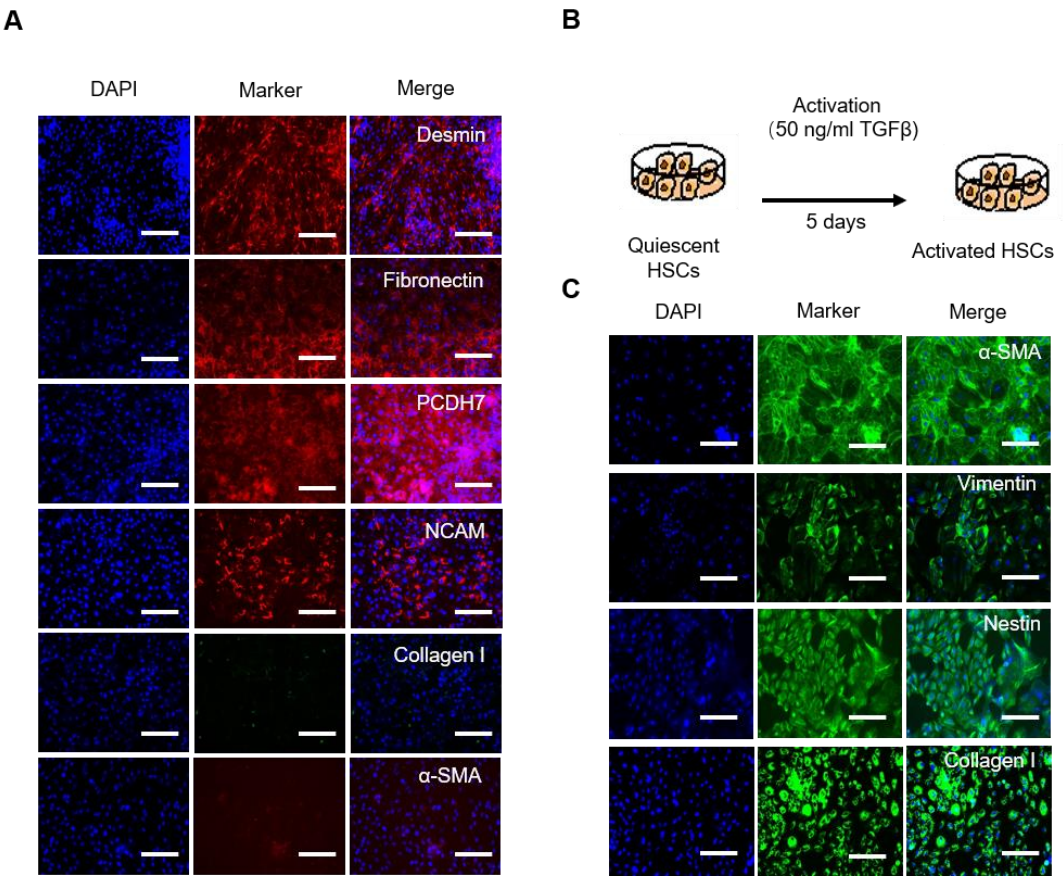
A
DAPI
Marker
Merge
Desmin
Fibronectin
PCDH7
NCAM
Collagen I
α-SMA
B
Activation
(50 ng/ml TGFβ)
5 days
Quiescent
HSCs
Activated HSCs
C
DAPI
Marker
Merge
α-SMA
Vimentin
Nestin
Collagen I

Figure S2. Characterization of iHSCs differentiated from hESCs (H1). Related to Figure 4. (A) Representative images of endoderm derived iHSCs markers expression. Scale bars, 100 μm. (B) Schematic representation of the activation of the iHSCs by TGFβ (50 ng/ml). (C) Representative images of activated iHSCs markers expression. Scale bars, 100 μm.

Figure S3

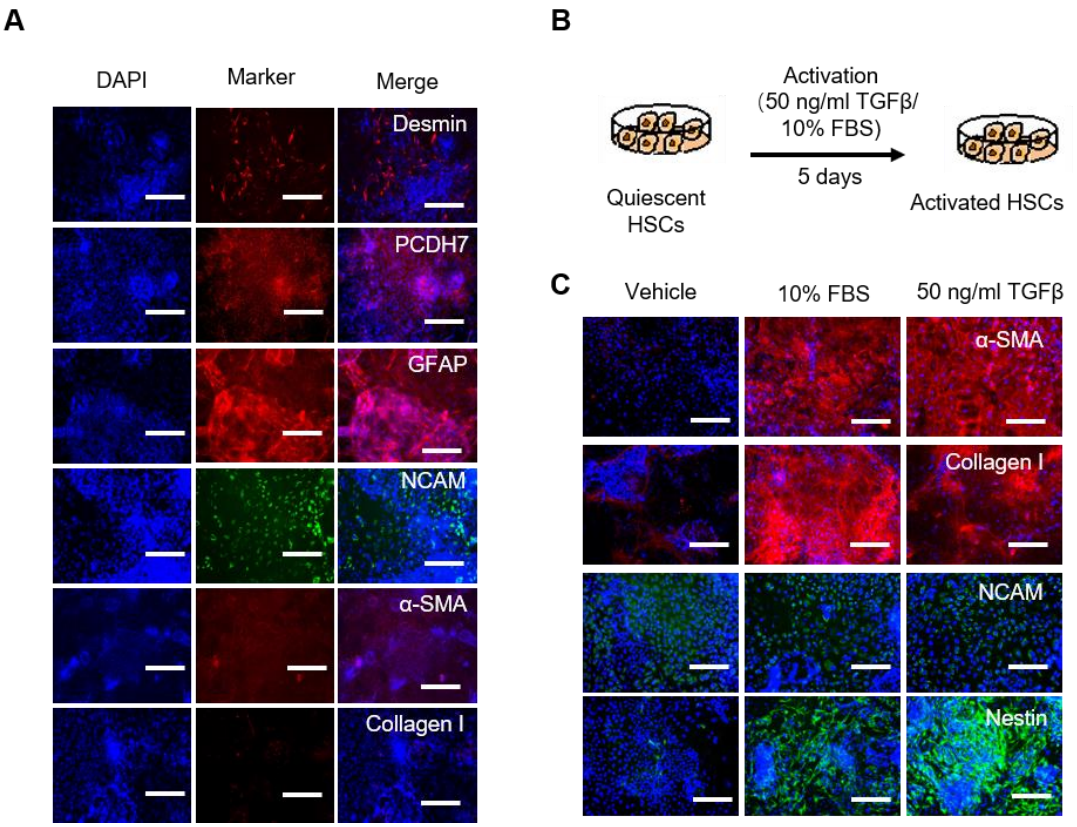
A
DAPI
Marker
Merge
Desmin
PCDH7
GFAP
NCAM
α-SMA
Collagen I
B
Activation
(50 ng/ml TGFβ/
10% FBS)
5 days
Quiescent
HSCs
Activated HSCs
C
Vehicle
10% FBS
50 ng/ml TGFβ
α-SMA
Collagen I
NCAM
Nestin

Figure S3. Characterization of iHSCs differentiated from iPSCs. Related to Figure 4. (A) Representative images of endoderm derived iHSCs markers expression. Scale bars, 100 μm. (B) Schematic representation of the activation of the iHSCs by TGFβ (50 ng/ml) and 10% FBS. (C) Representative images of activated iHSCs markers expression. Scale bars, 100 μm.

Figure S4

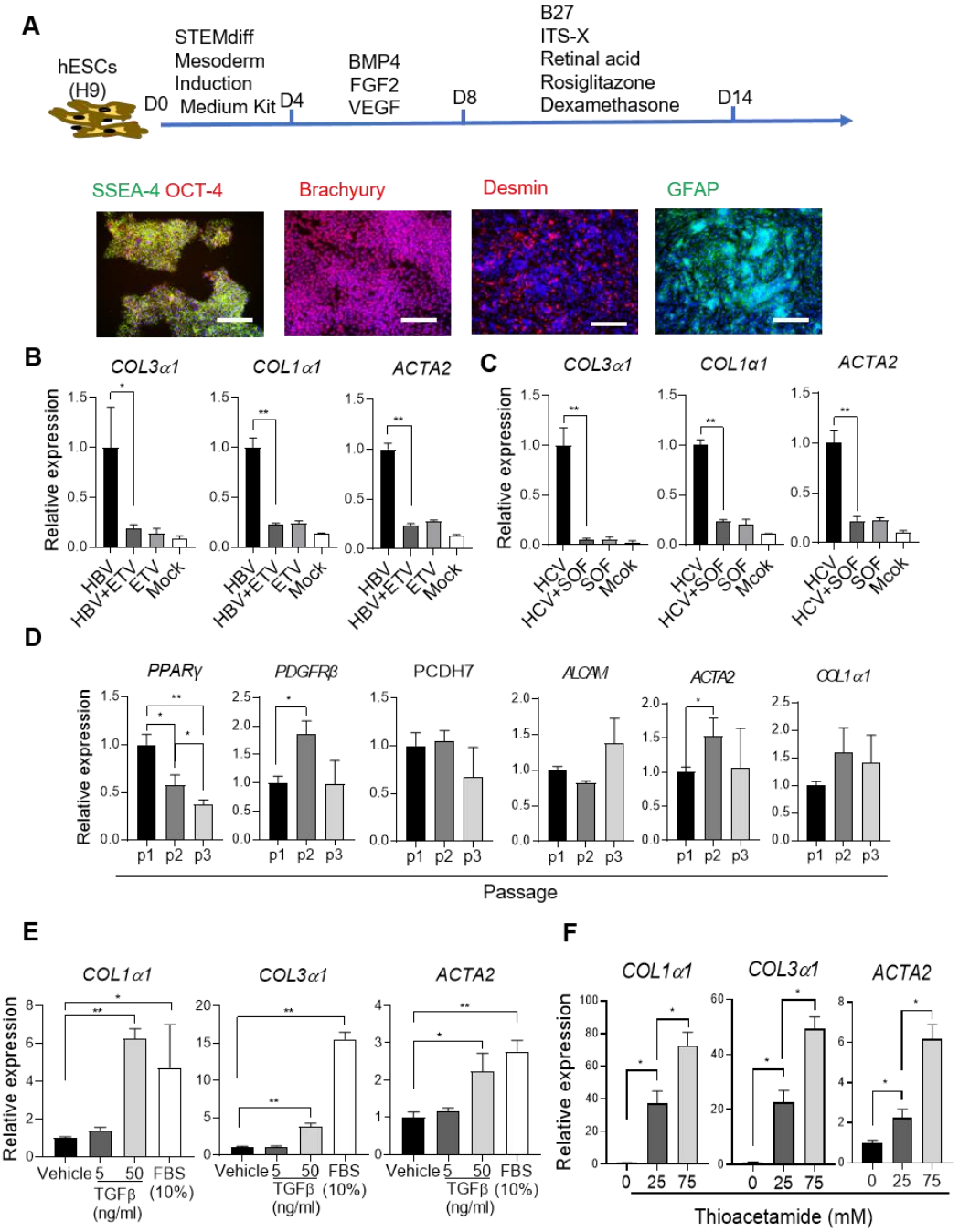
A
hESCs
(H9)
D0
STEMdiff
Mesoderm
Induction
Medium Kit
D4
BMP4
FGF2
VEGF
D8
B27
ITS-X
Retinal acid
Rosiglitazone
Dexamethasone
D14
SSEA-4 OCT-4
Brachyury
Desmin
GFAP
B
COL3α1
COL1α1
ACTA2
Relative expression
HBV
HBV+ETV
ETV
Mock
C
HCV
HCV+SOF
SOF
Mcok
D
PPARγ
PDGFRβ
PCDH7
ALCAM
ACTA2
COL1α1
p1
p2
p3
Passage
E
Vehicle
5
50
FBS
TGFβ (ng/ml)
(10%)
F
Thioacetamide (mM)
0
25
75

Figure S4. Characterization of mesoderm derived iHSCs from hESCs (H9). Related to Figure 7. (A) Representative images of hESCs, mesoderm and iHSCs related markers during the different stage of iHSCs differentiation according to the protocol shown above. Scale bars, 100 μm. (B) RT-qPCR analysis of *COL3α1*, *COL1α1* and *ACTA2* expression in the co-culture of iHSCs and HBV (500 geq/cell) infected HepG2-NTCP with or without entecavir (ETV) (500 nM) treatment for 7 days. (C) RT-qPCR analysis of *COL3α1*, *COL1α1* and *ACTA2* expression in the co-culture of iHSCs and HCV (MOI: 10) infected Huh7.5 cells with or without sofosbuvir (700 nM) for 3 days. (D) RT-qPCR analysis of gene expression in iHSCs at different passages. (E) RT-qPCR analysis of *COL1α1*, *COL3α1* and *ACTA2* expression of the second passaged iHSCs by the treatment of TGFβ and fetal bovine serum (FBS). (F) RT-qPCR analysis of *COL1α1*, *COL3α1* and *ACTA2* expression of the second passaged iHSCs by the treatment of different concentrations of thioacetamide. RT-qPCR data shown as the means±SEM from 3 independent experiments, n=3 in each group. Student's t test was used. *p<0.05.

Figure S5

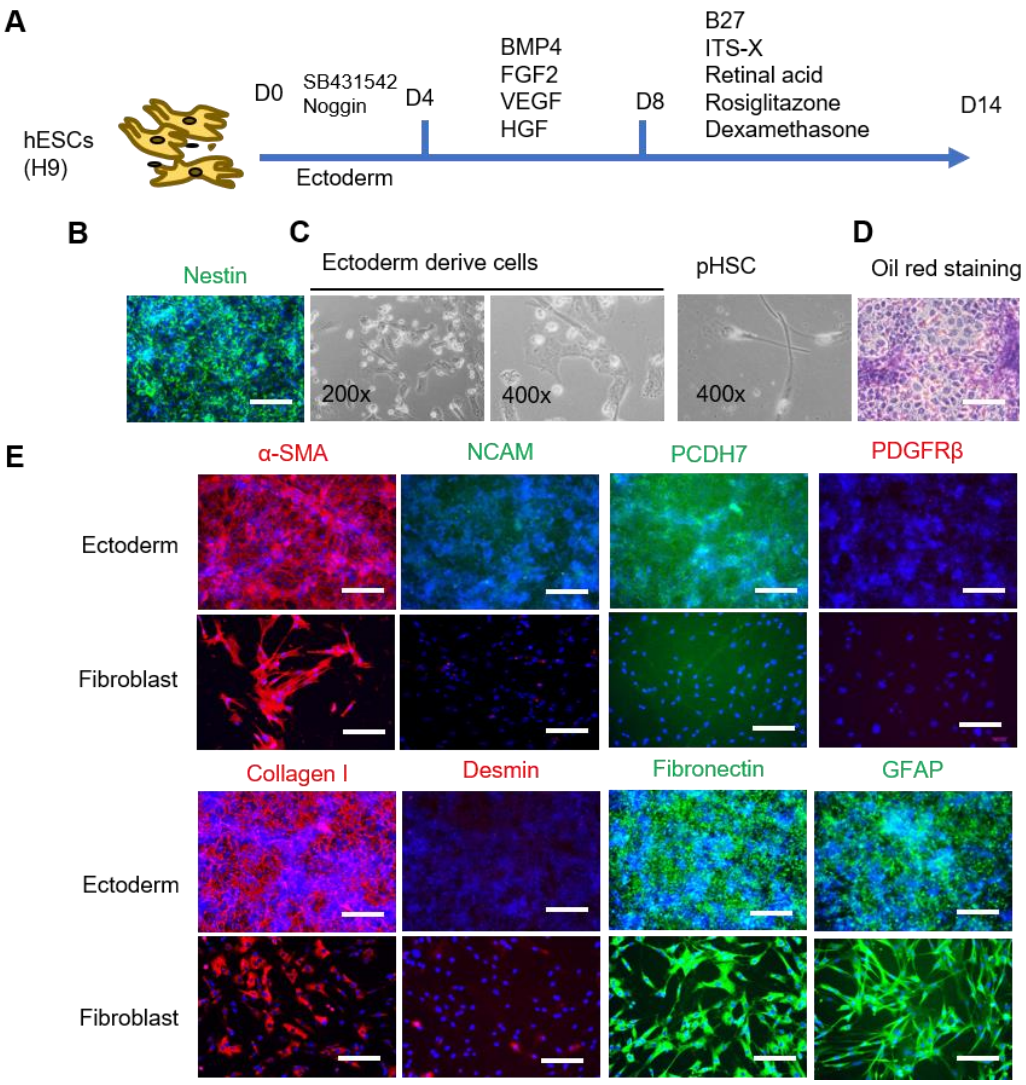
A
hESCs
(H9)
D0
SB431542
Noggin
D4
Ectoderm
BMP4
FGF2
VEGF
HGF
D8
B27
ITS-X
Retinal acid
Rosiglitazone
Dexamethasone
D14
B
Nestin
C
Ectoderm derive cells
200x
400x
pHSC
400x
D
Oil red staining
E
α-SMA
NCAM
PCDH7
PDGFRβ
Ectoderm
Fibroblast
Collagen I
Desmin
Fibronectin
GFAP
Ectoderm
Fibroblast

Figure S5. Characterization of ectoderm derived differentiation by the established differentiation protocol. Related to Figure 7. (A) Schematic representation of the differentiation process from day 0 to day 14. (B) Representative image of nestin expresion in ectoderm. Scale bars, 100 μm. (C) Representative images of cell morphology on day 14 differentiation. (D) Oil red staining analysis of lipid droplet in ectoderm derived cells. (E) Representative images of ectoderm derived cells and fibroblast cells. Scale bars, 100 μm.

Supplementary Tables

Table S1 Top downregulated genes in iHSCs compared to other cells. (See the excel file)

Table S2 Antibodies used for immunofluorescence assay and flow cytometry.

Protein	Supplier	Dilution
α-SMA	Abcam	1/500
ALCAM	Abcam	1/500
β-Actin	Cell Signaling	1/500
Collagen I	Abcam	1/500
Desmin	Abcam	1/500
Fibronectin	Sigma	1/500
NCAM	Sigma	1/500
NGF	Santa Cruz	1/500
PDCH7	Abcam	1/500
PDGFRβ	Abcam (Cambridge. UK)	1/500
Vimentin	Sigma	1/500
SSEA-4	Stem cell	1/500
Nestin	R&D Systems	1/500
Sox17	R&D Systems	1/500
Brachyury	CST	1/500
GFAP	R&D Systems	1/500
AFP	Sigma	1/500

Table S3. List of primers.

Gene	Forward (5'→3')	Reverse (5'→3')
GAPDH	AGCCACATCGCTCAGACAC	GCCCAATACGACCAAATCC
RPS11	GCCGAGACTATCTGCACTAC	ATGTCCAGCCTCAGAACTTC
COL1α1	GACACAGAGGTTTCAGTGG	CACCCTTAGCACCAACAG
COL3α1	GGAGCTGGCTACTTCTCGC	GGGAACATCCTCCTTCAACAG
ALB	TGGCACAATGAAGTGGGTAA	CTGAGCAAAGGCAATCAACA
PDGFRα	AACCCTGCTGATGAAAGCAC	TCCTTTCTAGCATGGGGACA
Desmin	AGGAACAGCAGGTCCAGGTA	AGAGCATCAATCTCGCAGGT
GFAP	GGATGGAGAGGTCATTAAGGA	GGTGAGTTTCTTGTTAGTTGGA
PDGFRβ	CCCTTATCATCCTCATCATGC	CCTTCCATCGGATCTCGTAA
Oct-4	GATGGCGTACTGTGGGCCC	TGGGACTCCTCCGGGTTTTG
ALCAM	ACCTCAGAATCTCATGTTTGG	GTTTAGATGGTTGCTTGAACAC
ACTA2	CCAGAGCCATTGTCACACAC	CAGCCAAGCACTGTCAGG
NCAM	AGGAGACAGAAACGAAGCCA	GGTGTTGGAAATGCTCTGGT
FOXA2	ATTGCTGGTCGTTTGTTGTG	TACGTGTTCATGCCGTTCAT
SOX17	CGCACGGAATTTGAACAGTA	GGATCAGGGACCTGTCACAC
hSTAP	TGCCAGTGACTTCCCACCT	TAGATGAGGCCACGCAGC
AFP	AGA CTG AAA ACC CTC TTG AAT GC	GTC CTC ACT GAG TTG GCA ACA
PCDH7	GGATCGGGTGAGGTGACTTTC	GTTCTCGTCGAAGATCATCTGAC
THY-1	AAGGACGAGGGCACCTACAC	GAGGTGTTCTGAGCCAGCAG
PPARγ	GAGGAGAGTTACTTGGTCGT	CAACAGACAAATCACCATTCG

Supplementary Experimental Procedures

Immunofluorescence staining

The immunofluorescence staining for related markers expression was performed as previously described (Coll et al., 2018). Briefly, cells were fixed in 4% para-formaldehyde in phosphate-buffered saline (PBS) at room temperature for 10 min and permeabilized with 0.1% Triton X-100 in PBS. Then, the cells were blocked with blocking buffer [PBS containing 10% goat serum, 1% bovine serum albumin (BSA)] at room temperature for 2 h. Cells were incubated overnight at 4 ℃ with primary antibody diluted with blocking buffer. Secondary antibodies (1:1000 dilution) were incubated in dark at room temperature for 1 h. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) diluted in PBS at room temperature for 20 min. Antibodies and dilutions used are listed in Table S2.

Quantitative real-time RT-PCR

Total RNA was obtained by using the RNAeasy Mini Kit (QIAGEN) followed by reverse transcription with Superscript III First-Strand Synthesis System. Gene expression was determined by quantitative real-time PCR on ABI 7500 cycler using Powerup SYBR master mix (Life technologies). The gene expression was normalized to RPS11 and calculated with the comparative Ct method ($2^{-\triangle\triangle Ct}$). Primers are listed in Table S3.

Flow cytometry

Differentiated cells were dissociated into a single-cell suspension and stained with antibodies for COT4, SSEA4, Brachyury, PDGFRβ, PDGFRα, Collagen I and α-SMA. Stained cells were analyzed with FACSCanto II cytomerter and FlowJo VX software. FACS plot showing populations were usually 10,000 and the positive gates were defined based on the isotype control.

Oil red staining

The differentiated cells at day 14 were analyzed for the vitamin A uptake by oil red staining. Cells were washed by DPBS for three times and fixed with 4% para-formaldehyde in PBS at room temperature for 10 min followed by 60% isopropanol washing. Then, cells were incubated with Oil Red (Sigma) working solution (6:4 Oil Red: ddH2O) for 30 min at room temperature. Finally, cells were counterstained with Gill's hematoxylin and washed with PBS. The images were performed by a fluorescence microscope (ECHO laboratories, USA)

Vitamin A storage assay

The vitamin A storage assay was performed as previously described. Briefly, the differentiated cells at day 14 were measured by the auto-fluorescence after UV light excitation and flow cytometry.

Genome wide transcriptome analysis of iHSCs

RNA-seq was performed at Novogene Co. Ltd. Total RNA was isolated from endoderm derived iHSCs (iHSC-end, n=3 independent differentiations), mesoderm derived iHSCs (iHSCs-M_2, n=3 independent differentiations), fibroblast (n=3 independent fibroblast cells), purchased primary HSCs (purHSC, n=3 independent donors cells bought from Lonza) and freshly pHSCs isolated from liver transplantation patients (n=3 different liver donors). Total RNA was isolated by using the RNeasyMini Kit (QIGEN). RNA sequencing libraries were prepared by using the NEBNext UltraTM RNA library Prep kit for Illumina (NEB) following the manufacturer's instructions. The randomly primed 150-bp paired-end libraries were sequenced on Illumina Hiseq 4000 platform. Raw data for human quiescent HSC (Liu et al., 2020) (qHSCs, GSE141100), hepatocyte (Jacquemin et al., 2013) (hep, GSE43984) and cultured hepatocytes (Koui et al., 2017) (cul_hep, GSE43984) and the iPSC-derived qHSC like cells (Koui et al., 2021) (TkDN4_M_qHSCs and FF-1_qHSCs, GSE155017) were extracted from GEO. The reads were aligned to the human genome (hg38) using STAR v2.7.9a (Dobin et al., 2013) and gene expression levels were estimated using featureCounts (Liao Y et al, 2014). DESeq2 (Love et al., 2014) was used to perform differential gene expression analysis. The HSC gene set signature previously described (Coll et al., 2018) was used to perform a gene set enrichment analysis (GSEA). DAVID bioinformatics Resources 6.8 R package clusterProfiler was used to perform gene ontology (GO) and KEGG pathway analysis (Wu et al., 2021). Principal component analysis (PCA) was generated with R PCA function using scaled gene expression. Heatmaps were generated by the pheatmap package.

Transwell co-culture of HepaRG with iHSCs or pHSCs

HepaRG cells were cultured in the HepaRG culture medium (Biopredic). Differentiated HepaRG cells (60,000 cells/well) were seeded in the insert of the transwell cultured in the Serum-free HepaRG medium. At the same time, the endoderm and mesoderm derived iHSCs (80,000 cells/well) were cultured in the plates. Then, the inserts were transferred in the iHSCs or pHSCs wells to form HepaRG co-cultured with iHSCs. As a positive control, iHSCs were replaced by primary human HSCs.

Exposure of iHSCs to acetaminophen and thioacetamide

Differentiated iHSCs cells were seeded at 80,000 cells per well in the Matrigel coated 24-well plate. Acetaminophen and thioacetamide at the concentration of 0, 25 and 75 mM were added to treat the cells for 48 h. The cells were harvested to analyze the activation of iHSCs by hepatotoxin exposure. Cell viability assay was performed by CellTiter-Blue assay as manufacturer's instructions.

Exposure of co-cultured iHSCs/pHSCs and HepaRG to acetaminophen

Differentiated HepaRG cells (60,000 cells/well) were seeded in the insert of the transwell cultured in the serum-free HepaRG medium. The bottom of the transwell plate was seeded with the iHSCs or pHSCs (80,000 cells/well). The cells were put in a well for co-culture. The cells were exposed to the 0, 25 and 75 mM of acetaminophen for 48 h. RT-qPCR assay was performed to analyze the mRNA levels of HSCs activated

markers. Cell viability assay was performed by CellTiter-Blue assay as manufacturer's instructions (Coll et al., 2018).

Exposure of iHSCs to HBV infected HepG2-NTCP cells

HepG2-NTCP cells (60,000 cells/well) were seeded in the insert of the transwell plate and infected with HBV (500 geq/cell) for 24 h. The next day, the cells were washed with PBS and replenish with fresh serum-free differentiation medium and co-cultured with iHSCs (80,000 cells/well) respectively for seven days. 500 nM of entecavir (ETV) was used as control for inhibition of HBV replication (Michailidis et al., 2017).

Exposure of iHSCs to HCV infected Huh7.5 cells

Huh7.5 cells (60,000 cells/well) were seeded in the insert of the transwell plate and infected with HCV (JFH1, MOI:10) for 24 h. The next day, the cells were washed with PBS and replenish with fresh serum-free differentiation medium and co-cultured with iHSCs (80,000 cells/well) respectively for three days. The 700 nM of sofosbuvir (SOF) was used as control for inhibition of HCV replication (PascalMutz et al., 2018).

***In vitro* activation of iHSCs**

Differentiated cells at day 14, iHSCs were seed with 60 thousand per well in the 24-well plate and activated by incubating with Transforming Growth factor β (TGFβ) (5 ng/ml or 50 ng/ml) or FBS (10%) for different days as designed. RT-qPCR was used to evaluated stellate cells activation related gene expression after different days of

incubation.

In vitro wound-healing assay

After performed a scratch, the differentiated cells and primary HSCs were stimulated with 25 ng/ml PDGF-BB (Sigma) and 10% FBS for 24h. Images were captured with a fluorescence microscope (ECHO laboratories, USA). The wound closure distances were measured using ImageJ software.

Measurement of secreted collagen I

The secreted collagen I was detected by the chemiluminescence immunoassay (CLIA) kit according to the manufacturer's instructions (Ding Sheng Xing Ye Co.) detected by manufacturer-provided luminometer.

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