Supplementary Material

Human Adipose Derived Stem Cells Exhibit Enhanced Liver Regeneration in Acute Liver Injury by Controlled Releasing Hepatocyte Growth Factor

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Supplemental material and method

Fluorescence-activated cell sorting (FACS) analysis

FACS analysis was performed as previously reported[1]. Cells were resuspended in Dulbecco's PBS (Cellgro, Manassas, MD) and incubated for 20 min at 4°C either directly with PE- or with FITC-conjugated Abs. The antibodies used were anti-CD3, anti-CD11b, anti-CD14, anti-CD19, anti-CD29, anti-CD31, anti-CD34, anti-CD44, anti-CD45, anti-CD73, anti-CD90, anti-CD105, anti-CD133 and anti-HLA-DR (all from BD). The corresponding isotype-identical IgGs served as negative controls. After cell staining, quantitative FACS was performed on a FACStar flow cytometer (BD).

Lentiviral vector production and transduction

293T cells (6×10^6) were seeded in 10 cm^2 plates. After 24 h, the cells were transfected with the following plasmids using Lipofectamine 3000 (Invitrogen): pMDLg/p RRE (Gag-Pol expression plasmid), pCMV-VSV-G-RSV-Rev (Rev and VSV-G env expression plasmid), or pLV-EF1-GFP-Neo. Forty-eight hours later, the lentivirus-containing supernatant was collected and filtered through 0.45 μm filters. Viral supernatants were used to infect ASCs that had been seeded in 75 cm^2 flasks at a density of 7×10^5 cells per flask. The cells were infected with 10 ml of virus in the presence of 100 μg/ml protamine sulfate (Sigma). Three days after infection, the infected cells were detected by fluorescence microscopy (**Supplementary Figure 2F**).

Microarray data analysis

RNA purity and integrity were evaluated using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, USA) and an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, USA). RNA labeling and hybridization were performed using the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). Briefly, 100 ng of total RNA from each sample was linearly amplified and labeled with Cy3-dCTP. The labeled cRNAs were purified using an RNAeasy Mini Kit (Qiagen). The concentration and specific activity of the labeled cRNAs (pmol Cy3/μg cRNA) were measured using a NanoDrop ND-1000 (NanoDrop, Wilmington, USA). In total, 600 ng of each labeled cRNA was fragmented by adding 5 μl of 10x blocking agent and 1 μl of 25x fragmentation buffer and then heated at 60°C

for 30 min. Finally, 25 μl 2x GE hybridization buffer was added to dilute the labeled cRNA. In total, 40 μl of hybridization solution was dispensed into the gasket slide and assembled with the Agilent SurePrint G3 Human GE 8X60K, V3 Microarrays (Agilent®). The slides were incubated for 17 h at 65°C in an Agilent hybridization oven and then washed at room temperature using the Agilent One-Color Microarray-Based Gene Expression Analysis protocol (Agilent Technology, V 6.5, 2010). The hybridized array was immediately scanned with an Agilent Microarray Scanner D (Agilent Technologies, Inc.) Raw data were extracted using the Agilent Feature Extraction Software (v11.0.1.1). The raw data for the same gene were then summarized automatically by the Agilent feature extraction protocol to generate a raw data text file, providing expression data for each gene probed on the array. Array probes that had a Flag A in the samples were filtered out. The selected processed signal value was transformed by logarithm and normalized by the quantile method. The statistical significance of the expression data was determined using fold change and the LPE test, in which the null hypothesis was that no difference exists among 2 groups. For the DEG set, hierarchical cluster analysis was performed using complete linkage and the Euclidean distance as a measure of similarity. Gene enrichment and functional annotation analysis for the significant probe list was performed using Gene Ontology (GO) (www.geneontology.org/) and KEGG (http://kegg.jp) (**Supplementary Figure 7**). All data analyses and visualization of differentially expressed genes were conducted using R 3.1.2 (www.r-project.org).

RT-PCR primers

The primers used for RT-PCR were as follows:

For mouse;

MMP-9-F	372bp	AACCTCCAACCTCACGGACA
MMP-9-R	372bp	CGCGACACCAAACTGGATGA
MMP-13-F	449bp	ACCTCCACAGRRGACAGGCT
MMP-13-R	449bp	AAGCTCATGGGCAGCAACAA
GAPDH- F	184bp	GTTGTCTCCTGCGACTTCA
GAPDH- R	184bp	GGTGGTCCAGGGTTTCTTA

Immunocytochemistry.

Cells were cultured in 2-well glass slides at 5 × 10^6 cells per well for 5 days. As hepatocyte markers, antibodies against AFP (1:400; Abcam Inc., Cambridge, MA, USA), ALB (1:300; Abcam), CK18 (1:200; Abcam), CK19 (1:200; Abcam) and HGF (1:200; Abcam) were used. In the fluorescent immunocytochemistry, Cy2- or Cy3-conjugated secondary antibodies were applied as appropriate.

References

1 Kim, M. H., Zhang, H. Z. & Kim, S. W. Combined growth factors enhanced angiogenic potential of cord blood-derived mononuclear cells transplanted to ischemic limbs. *Journal of molecular and cellular cardiology* **51**, 702-712, doi:10.1016/j.yjmcc.2011.07.006 (2011).

Supplementary Figure Legends

Supplementary Figure 1. HGF expression in ASCs after cytokine stimulation and its intracellular signaling pathway. (A) Enhanced HGF expression after stimulation with four cytokines. Cocktails are included with 20 ng/ml VEGF-A, 100 ng/ml FGF-2, 20 ng/ml EGF, and 20 ng/ml IGF-1. (B) FGF-2 concentration and enhanced HGF expression in ASCs. Cells were treated with FGF-2 for 6 hr. (C) Effects of down-stream signaling inhibitors on HGF mRNA expression. Three inhibitors for each signaling pathways were added at $10\mu M$ with FGF-2 (50ng/ml) for 24 hr. FGF-2 induced HGF expression was significantly inhibited by an ERK inhibitor (U0126, U), a c-Jun N-terminal kinase (JNK) inhibitor (SP600125, SP), and a p38 inhibitor (SB202190, SB). n = 5; **p < 0.01.

Supplementary Figure 2. Characteristics of ASCs and ASC-C. Cell morphology (A, C) and flow cytometry results of cell surface markers of ASCs and ASC-C (B, D). Isotype controls were overlaid in black on each histogram for specific surface antigens. (E) Quantification of flow cytometry data for ASCs and ASC-C. n = 3 per group. (G) GFP-expressing human ASCs used for a cell engraftment study in liver tissues.

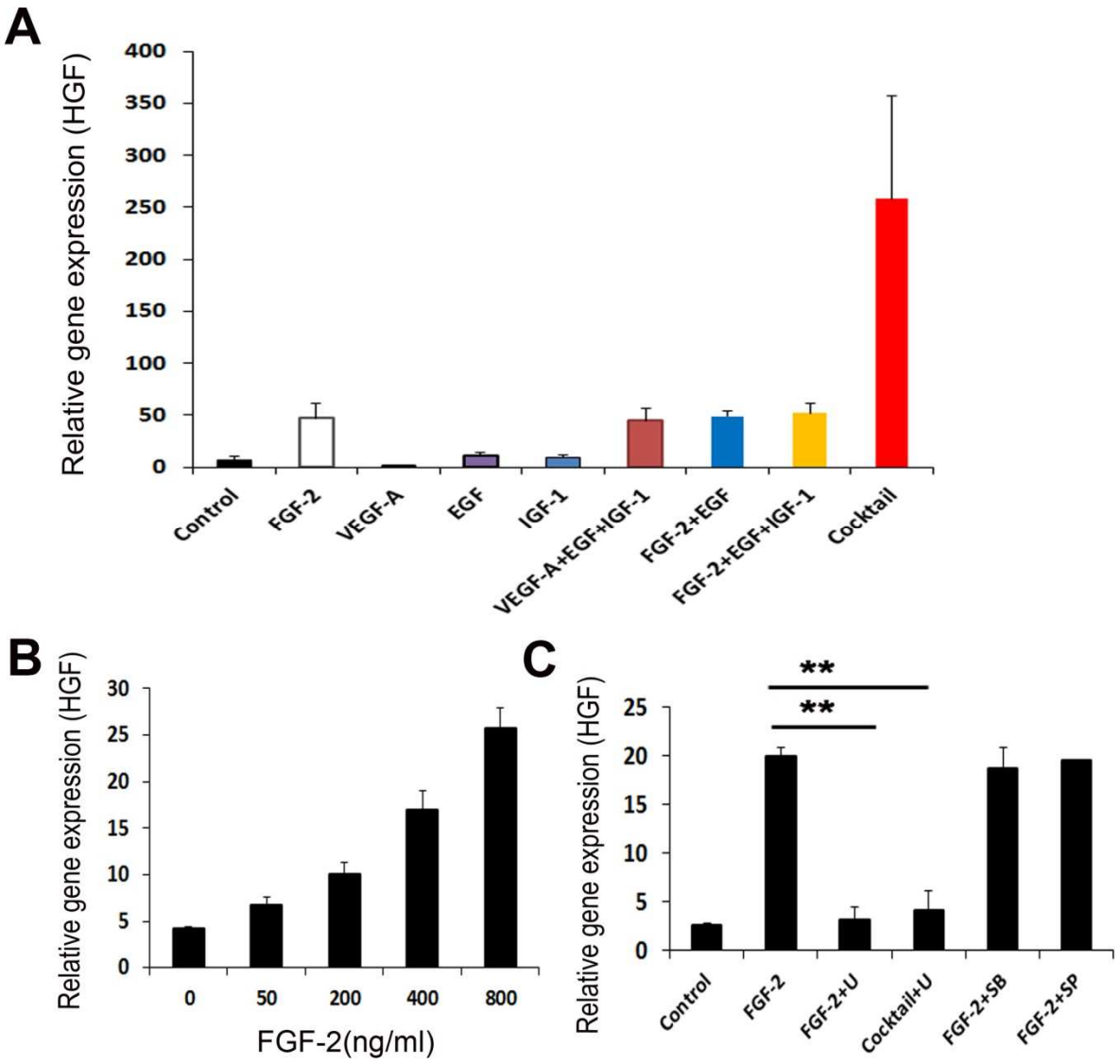
Supplementary Figure 3. Representative morphology of the liver after the induction of acute liver failure and stem cell injection. The surface contour of the livers derived from PBS- or ASCs-treated mice was markedly irregular compared with that of the ASC-C-treated mice.

Supplementary Figure 4. The analysis of ECM remodeling factors. The expression level of MMP-9 and -13 mRNAs in cell injected liver tissues were evaluated by RT-PCR and (D) intensity were quantified. n = 4 ; **p < 0.01, *p < 0.05.

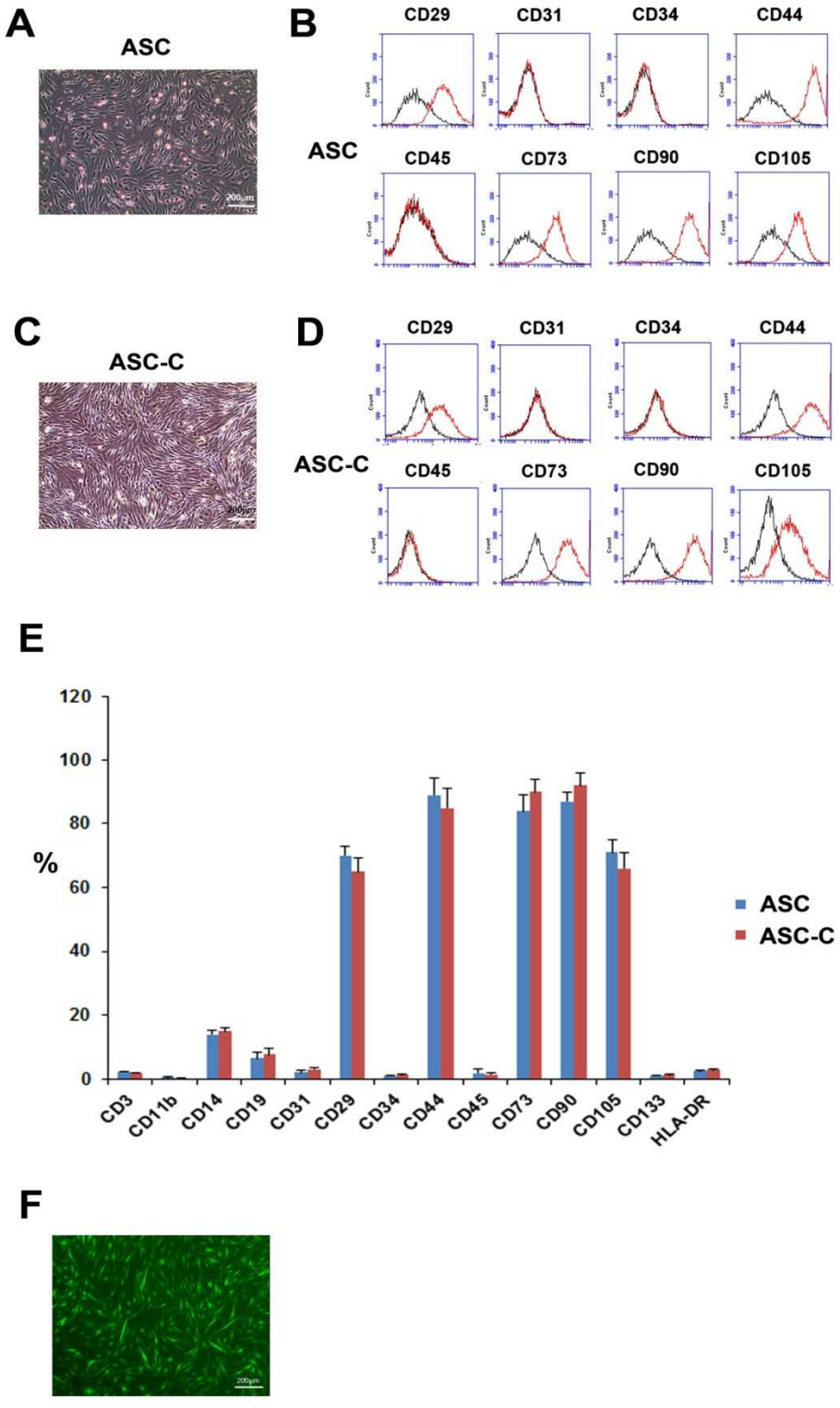
Supplementary Figure 5. ASC-C injection reduced apoptotic cells in injured liver tissue. Representative images of injured liver stained by terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) ((A) and anti-mouse albumin (C) and (B, D) quantitative analysis. n = 6; **p < 0.01. ALB, anti-mouse albumin; HPF, high power field.

Supplementary Figure 6. *In vivo* hepatocyte-like cell transdifferentiation of ASC-C and analysis of ECM remodeling factors. Injected ASC-C were engrafted in mice liver and they were expressing CK-18 (A) and CK-19 (B) after 3 weeks after cell injection. Arrows indicate hepatocyte-like cell transdifferentiated ASC-C.

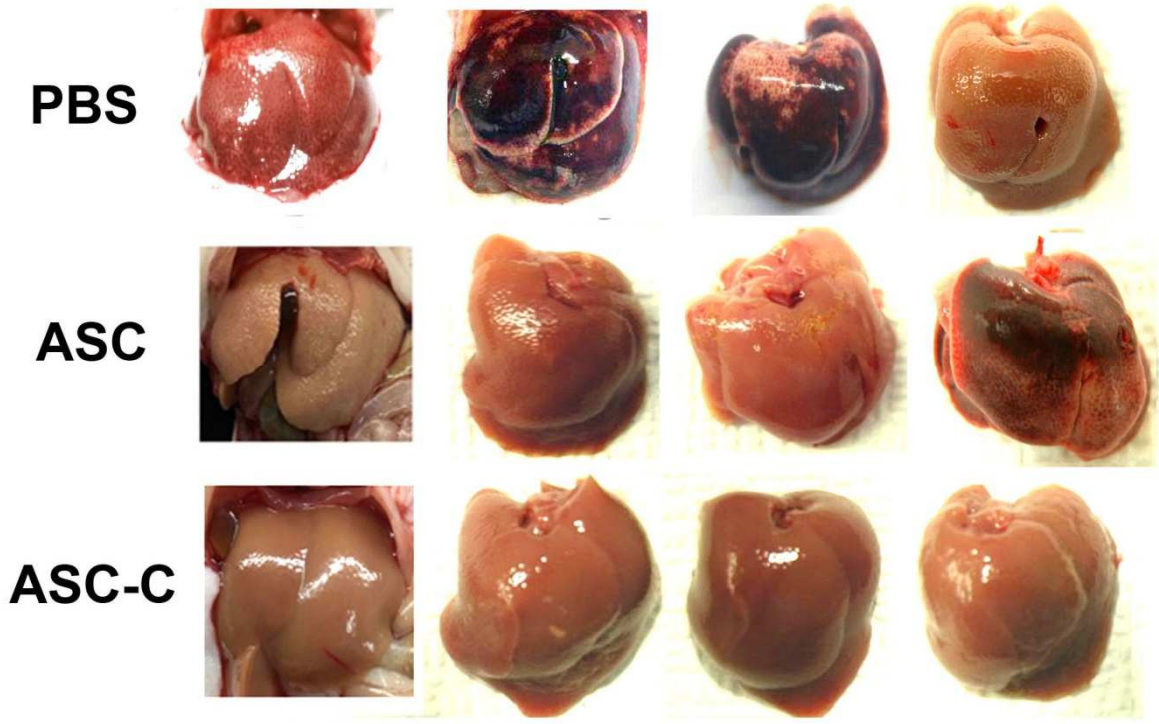
Supplementary Figure 1.



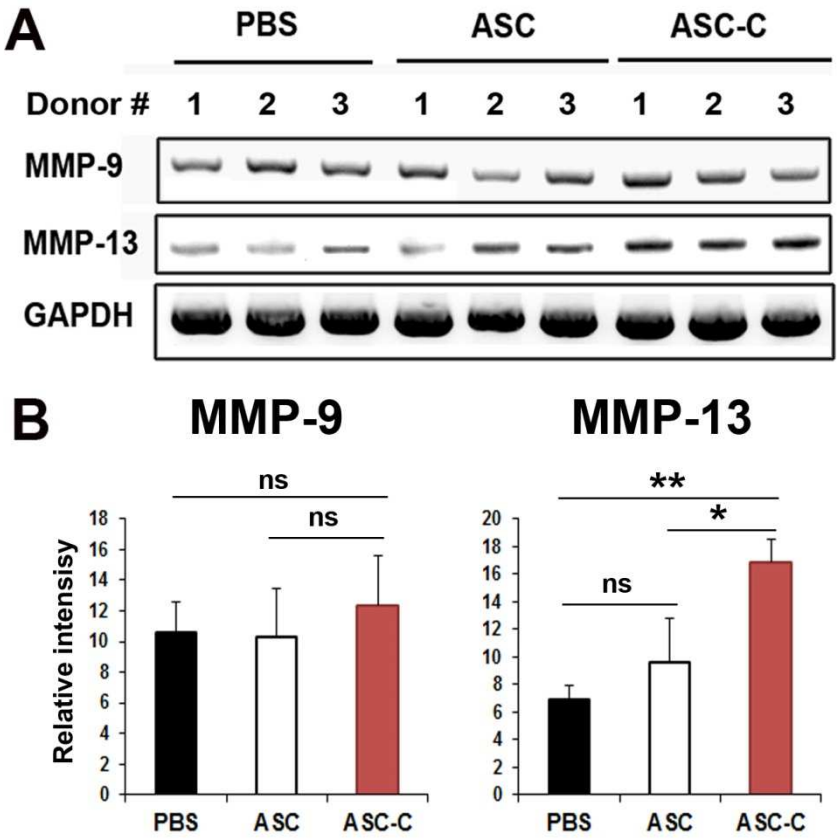
Supplementary Figure 2.



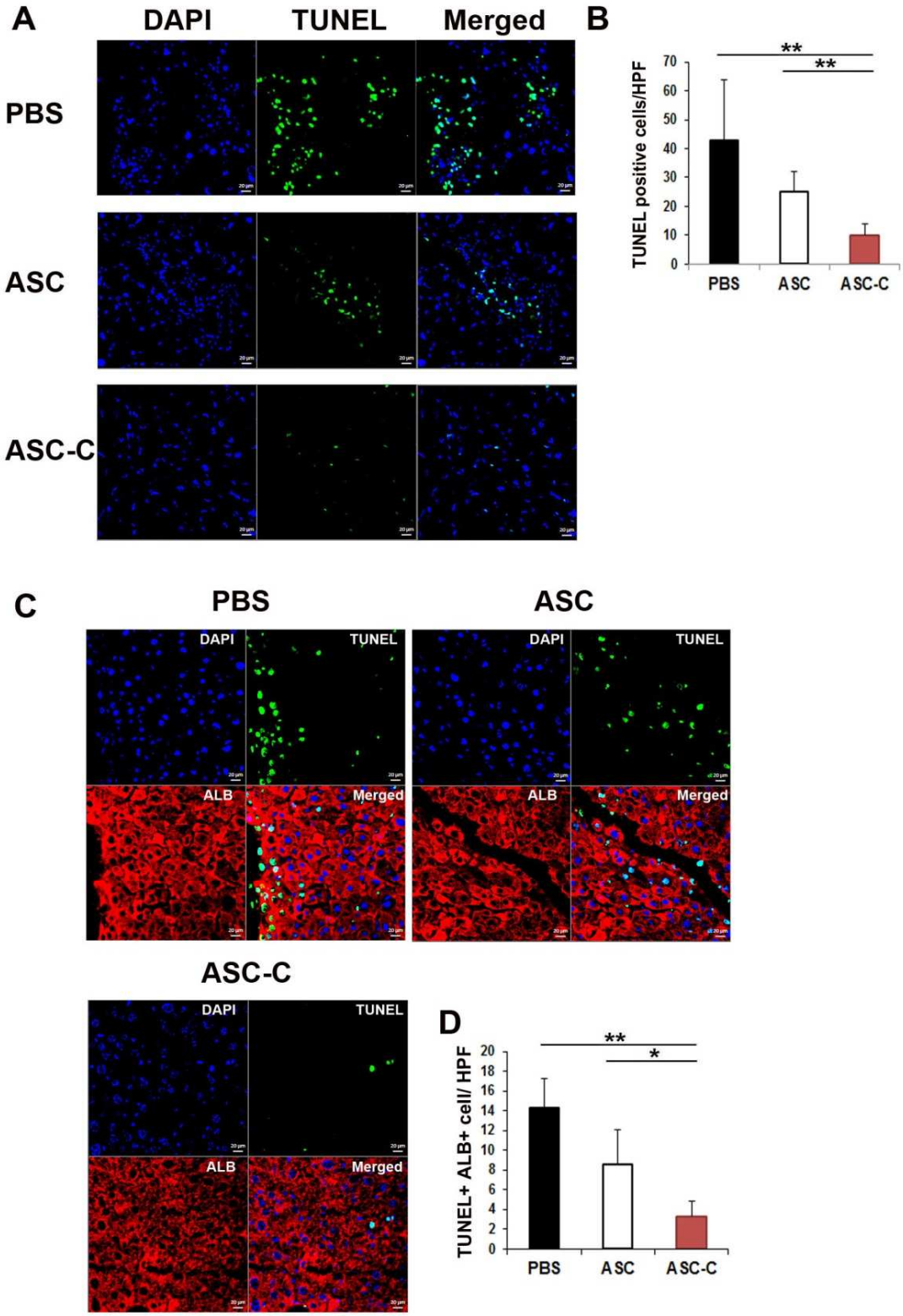
Supplementary Figure 3.



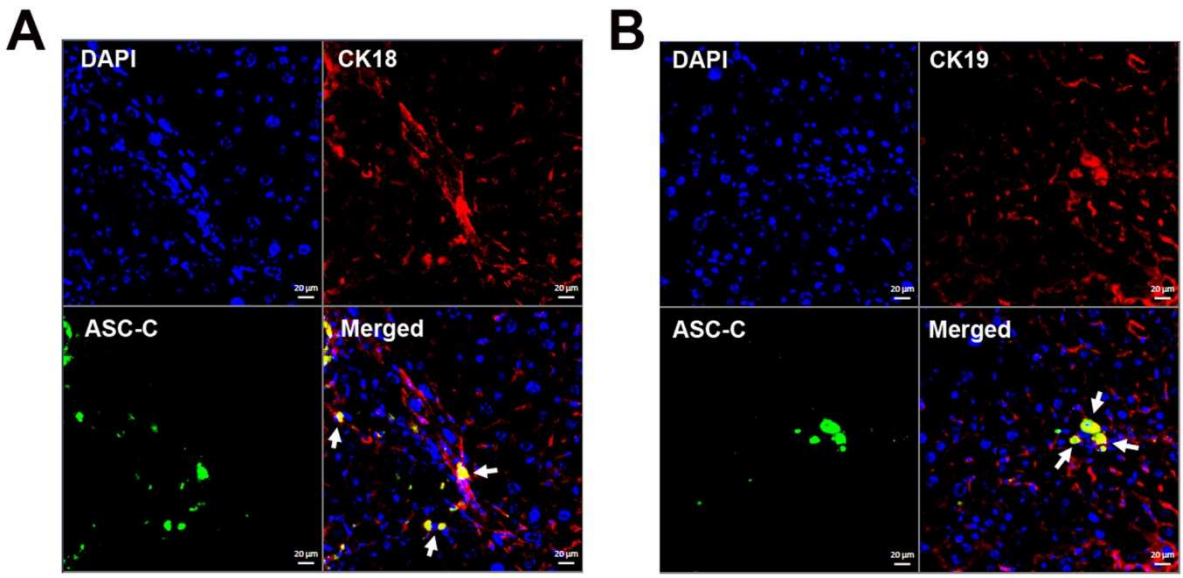
Supplementary Figure 4.



Supplementary Figure 5.



Supplementary Figure 6.



Supplementary Table 1. Top 50 up-regulated gene list in ASC-C compared with ASCs.

#	Probe ID	Gene_Symbol	Gene_ID	RefSeqAccession	Fold up-regulated
1	A_33_P3218649	GPM6B	2824	NM_001001995	**115.0**
2	A_23_P35725	ANO3	63982	NM_031418	**53.0**
3	A_23_P119943	IGFBP2	3485	NM_000597	**50.3**
4	A_24_P38081	FKBP5	2289	NM_004117	**48.7**
5	A_23_P149121	DIRAS3	9077	NM_004675	**45.5**
6	A_24_P64653	METTL7B	196410	NM_152637	**36.0**
7	A_33_P3281283	S1PR3	1903	NM_005226	**35.2**
8	A_23_P18372	B3GNT5	84002	NM_032047	**35.0**
9	A_23_P40880	CMTM8	152189	NM_178868	**34.5**
10	A_33_P3379061	C9orf47	286223	NM_001001938	**34.0**
11	A_23_P151710	PTGER2	5732	NM_000956	**29.2**
12	A_33_P3814721	INSC	387755	NM_001031853	**28.3**
13	A_33_P3276713	HGF	3082	NM_001010931	**26.1**
14	A_23_P501754	CSF3	1440	NM_000759	**23.3**
15	A_23_P167129	HHIP	64399	NM_022475	**23.1**
16	A_23_P371039	NTSR1	4923	NM_002531	**22.2**
17	A_22_P00013501	LOC100507065	100507065	NR_120431	**20.2**
18	A_22_P00000938	LINC00704	100216001	NR_024475	**20.1**
19	A_24_P79403	PF4	5196	NM_002619	**19.2**
20	A_33_P3415944	TMEM244	253582	NM_001010876	**18.2**
21	A_23_P106362	AQP9	366	NM_020980	**18.1**
22	A_23_P205428	FOXG1	2290	NM_005249	**18.0**
23	A_23_P259071	AREG	374	NM_001657	**17.6**
24	A_23_P104438	MYPN	84665	NM_032578	**17.3**
25	A_23_P165783	MLPH	79083	NM_024101	**16.7**
26	A_24_P183150	CXCL3	2921	NM_002090	**16.7**
27	A_23_P45365	COL4A5	1287	NM_033380	**16.1**
28	A_23_P159952	BEX1	55859	NM_018476	**15.8**
29	A_23_P136777	APOD	347	NM_001647	**15.7**
30	A_32_P32739	NAGS	162417	NM_153006	**15.6**
31	A_33_P3242543	MAOA	4128	NM_001270458	**15.6**
32	A_23_P115167	LRRC8B	23507	NM_015350	**14.9**
33	A_23_P133293	MCTP1	79772	NM_024717	**14.6**
34	A_23_P124905	NPTX1	4884	NM_002522	**14.5**
35	A_23_P125383	TMEFF2	23671	NM_016192	**14.2**
36	A_24_P307653	SLC6A15	55117	NM_182767	**14.2**
37	A_24_P416346	ETV4	2118	NM_001079675	**14.1**
38	A_19_P00322339	LINC00707	100507127	NR_038291	**14.0**
39	A_23_P17065	CCL20	6364	NM_004591	**13.8**
40	A_23_P70785	AIM1	202	NM_001624	**13.7**
41	A_33_P3842551	IKZF2	22807	NM_001079526	**13.7**
42	A_23_P413641	PREX1	57580	NM_020820	**13.1**
43	A_21_P0012715	LINC01085	152742	NR_033931	**13.0**
44	A_23_P104804	ZBTB16	7704	NM_006006	**13.0**
45	A_23_P148047	PTGER4	5734	NM_000958	**12.9**
46	A_23_P91636	POM121L9P	29774	NR_003714	**12.8**
47	A_23_P28466	DAW1	164781	NM_178821	**12.7**
48	A_24_P291658	ADH1A	124	NM_000667	**12.4**
49	A_33_P3351371	CYP19A1	1588	NM_031226	**12.4**
50	A_33_P3255304	GGT5	2687	NM_001099781	**11.5**

Supplementary Table 2. Top 15 up-regulated hepatocyte-related gene list in ASC-C compared with ASCs.

#	Probe ID	Gene Symbol	Gene ID	RefSeqAccession	Fold up-regulated
1	A_33_P3276713	HGF	3082	NM_001010931	**26.1**
2	A_23_P106362	AQP9	366	NM_020980	**18.1**
3	A_23_P205428	FOXG1	2290	NM_005249	**18.0**
4	A_33_P3351371	CYP19A1	1588	NM_031226	**12.4**
5	A_33_P3241511	SERPIND1	3053	NM_000185	**6.6**
6	A_33_P3355503	FOXL1	2300	NM_005250	**3.6**
7	A_33_P3408757	FOXO6	100132074	NM_001291281	**2.7**
8	A_33_P3298539	APOA1	335	NM_000039	**1.8**
9	A_23_P71855	C5	727	NM_001735	**1.8**
10	A_23_P161998	HPX	3263	NM_000613	**1.7**
11	A_23_P23996	MAT1A	4143	NM_000429	**1.6**
12	A_33_P3328312	HNF1A	6927	NM_000545	**1.3**
13	A_33_P3421243	AFP	174	NM_001134	**1.2**
14	A_23_P155123	CYP2D6	1565	NM_000106	**1.2**
15	A_23_P375372	FGA	2243	NM_021871	**1.2**