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Supplemental Information

CPM Is a Useful Cell Surface Marker to Isolate Expandable Bi-Potential Liver Progenitor Cells Derived from Human iPS Cells

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Supplemental Information

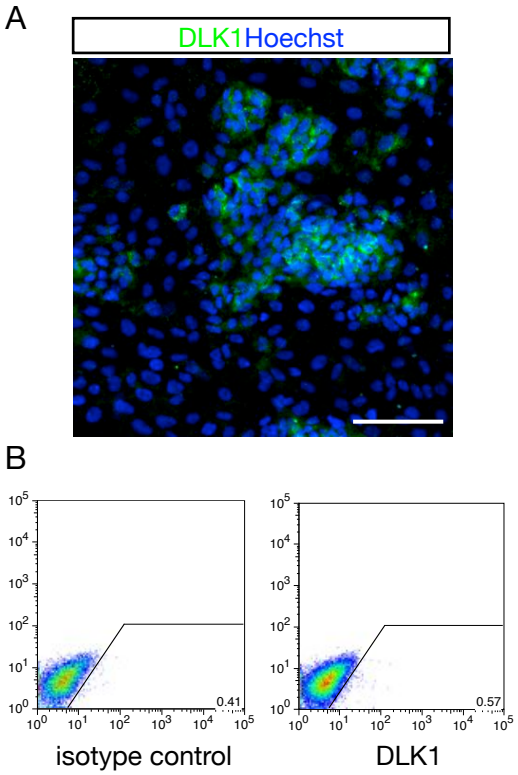

Figure S1. DLK1 expression during hepatic differentiation from hiPSCs, related to Figure 1

(A) Immunofluorescence analysis for DLK1 (green) in the hiPSC-derived immature hepatocyte stage. Nuclei were counterstained with Hoechst 33342 (blue). (B) FCM analysis for DLK1 expression.

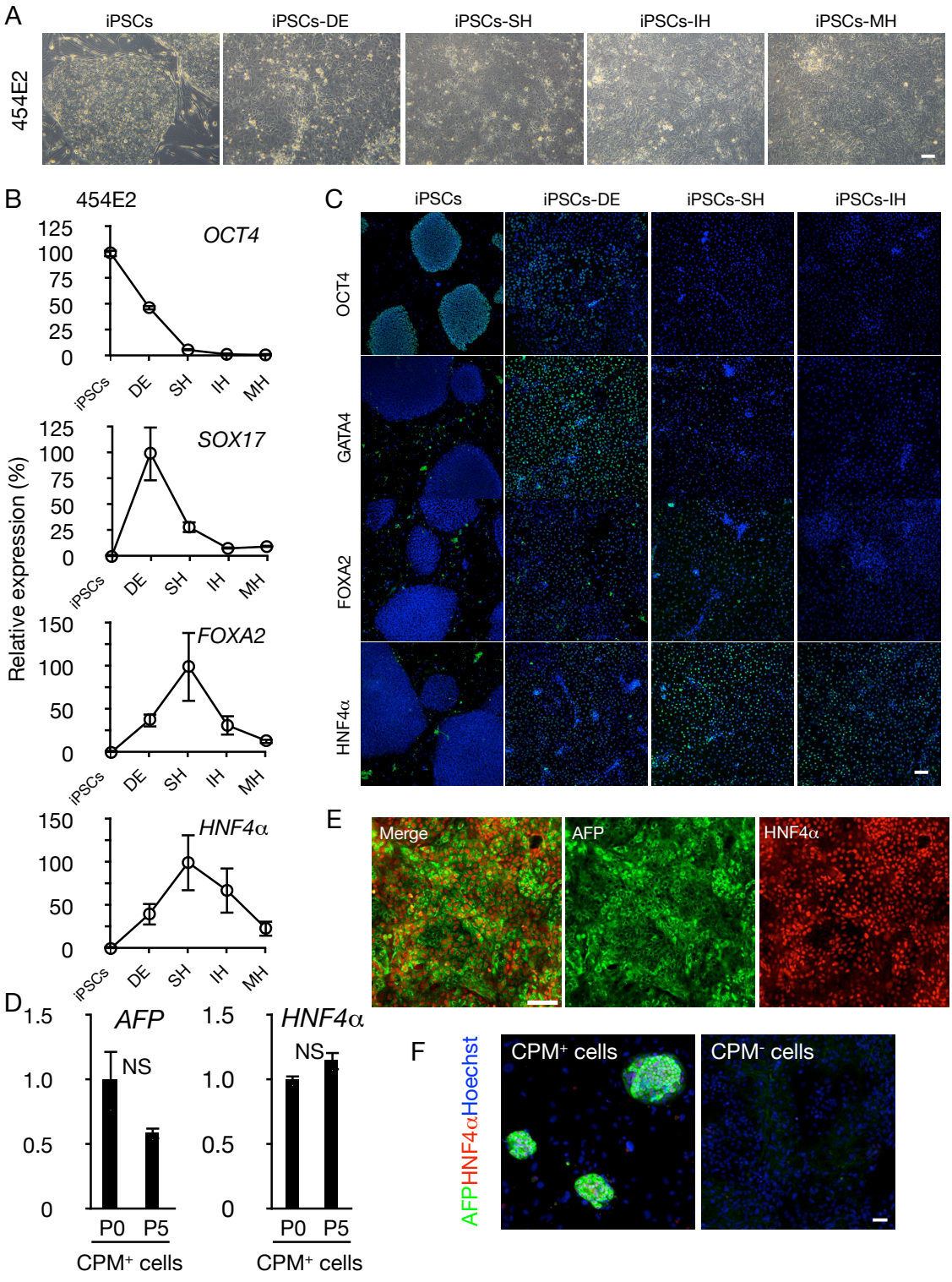

Figure S2. Induction of hepatic differentiation from hiPSCs, related to Figure 2

(A) Morphological changes of hiPSCs at different stages of hepatic differentiation. Scale bar, 100 μm. (B) Quantitative RT-PCR analysis for *OCT4*, *SOX17*, *FOXA2* and *HNF4α*. Error bar represents the mean ± SEM of 3 independent experiments. (each experiment contains 2 technical replicates) (C) Immunofluorescence analysis for OCT4, GATA4, FOXA2 and HNF4 α (green). Nuclei were counterstained with Hoechst 33342 (blue). Scale bar, 100 μm. (D) Expression of hepatoblast markers in CPM^+ LPCs. The results are shown as the mean ± SEM of 6 independent experiments. P0: passage 0, P5: passage 5. NS, no significance. (each experiment contains 2 technical replicates) (E) Immunofluorescence analysis for AFP (green) and HNF4 α (red) in CPM^+ LPCs after cryopreservation. (F) Immunofluorescence analysis shows the expression of AFP (green) and HNF4 α (red) in CPM^+ LPCs derived from 409B2 line. Nuclei were visualized by Hoechst 33342 staining (blue). Scale bar, 100 μm.

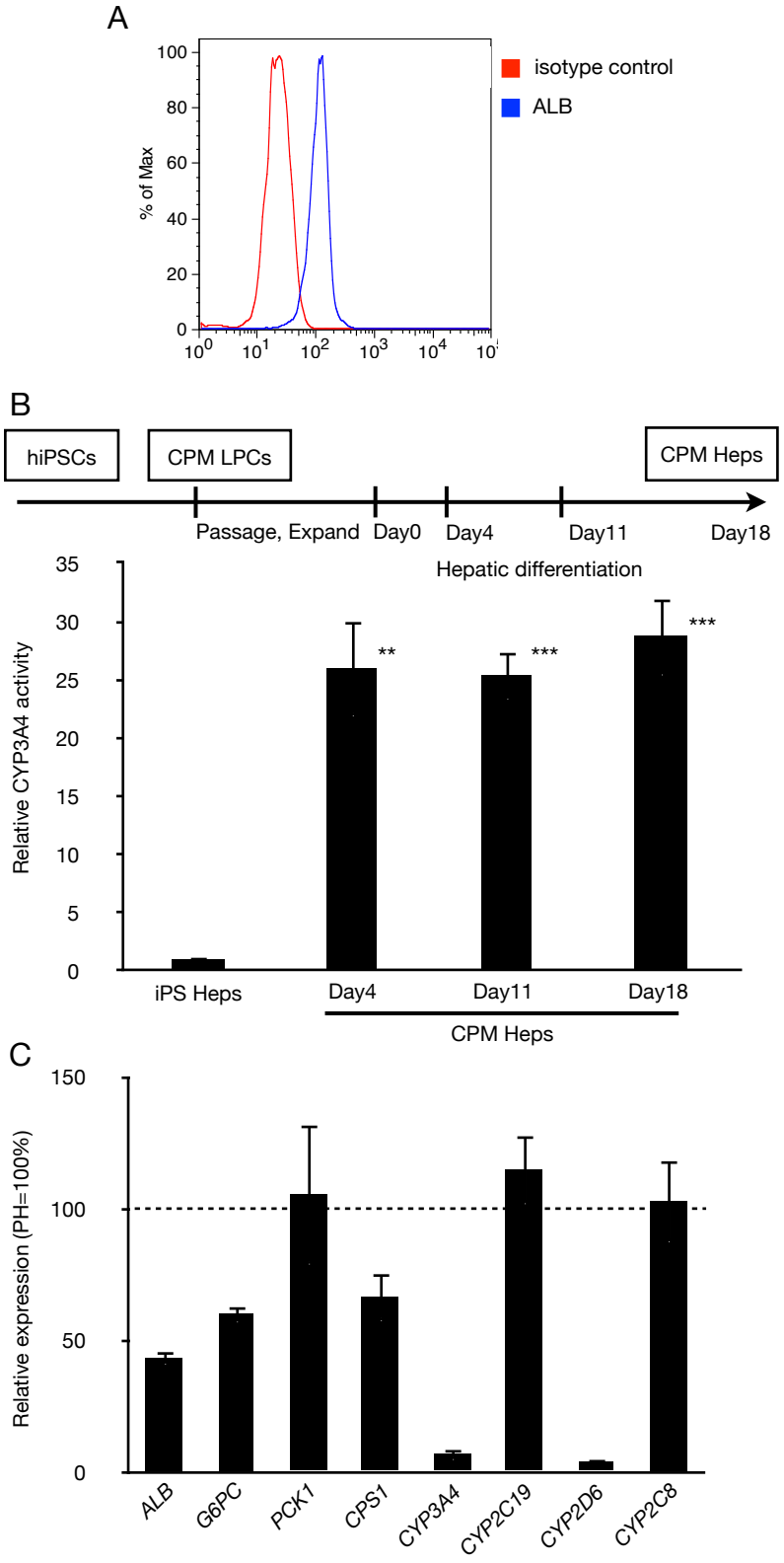

Figure S3. Differentiation of hepatocytes from CPM$^+$ LPCs, related to Figure 3

(A) FCM analysis for ALB. (B) CYP3A4 activities in the culture of CPM^+ hepatocytes at day 0, 4, 18. The results are shown as the mean ± SEM of independent experiments. n=8, 6, 6, 6 in each group. **P<0.01, ***P<0.001 (C) Relative *ALB*, *G6PC*, *PCK1*, *CPS1*, *CYP3A4*, *CYP2C19*, *CYP2D6* and *CYP2C8* expressions in the CPM^+ hepatocytes compared with primary human hepatocytes (PH). The results are shown as the mean ± SEM of independent experiments. n=6. (each experiment contains 2 technical replicates)

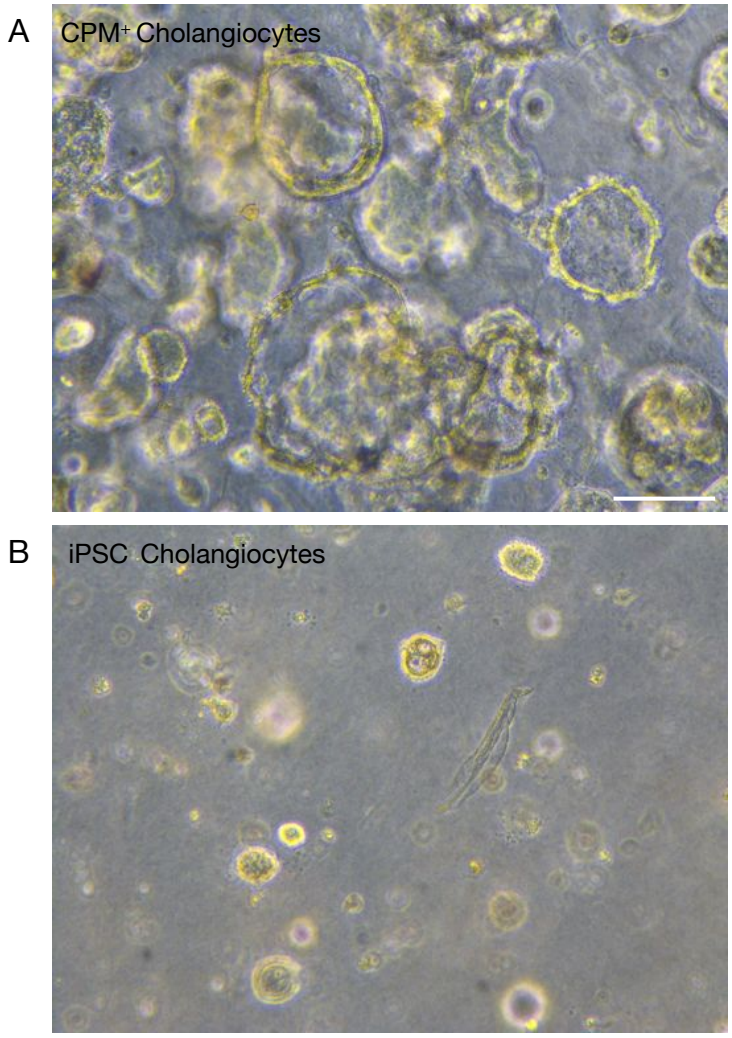

Figure S4. Differentiation of cholangiocytes from CPM$^+$ LPCs, related to Figure 4

(A, B) Phase contrast images. CPM$^+$ cholangiocytes (A) and iPSC cholangiocytes (without CPM purification process) (B). Scale bar, 100 μm.

Table S1. List of Quantitative RT-PCR primers for mouse and human genes, related to Figures 1-4, S2 and S3

	Left primer	Right primer	Product size
Actb	TTCTTTGCAGCTCCTTCGTT	ATGGAGGGGAATACAGCCC	149
Afp	GGCGATGGGTGTTTAGAAAG	CAGCAGCCTGAGAGTCCATA	95
Alb	TGCACACTTCCAGAGAAGGA	GTCTTCAGTTGCTCCGCTGT	98
Cpm	CCCGTTTAGAACCAACAAGC	GAGTCGTGTCCAGGGACTGT	78

	Left primer	Right primer	Product size
ACTB	GCACAGAGCCTCGCCTT	GTTGTCGACGACGAGCG	93
AFP	AGAGGAGATGTGCTGGATTG	GTGGTCAGTTTGCAGCATTC	110
ALB	TGCTGATGAGTCAGCTGAAAA	TCAGCCATTTCACCATAGGTT	105
OCT4	GAAGGAGAAGCTGGAGCAAA	CTTCTGCTTCAGGAGCTTGG	94
SOX17	CAGAATCCAGACCTGCACAA	TCTGCCTCCTCCACGAAG	101
FOXA2	CGACTGGAGCAGCTACTATGC	TGTTGCTCACGGAGGAGTAG	90
HNF4α	GCAGGCTCAAGAAATGCTTC	GGCTGCTGTCCTCATAGCTT	102
CPM	GGATGGAAGCGTTTTTGAAG	CCACAACAAGAACCCACAGG	108
CYP3A4	TTTTGTCCTACCATAAGGGCTTT	CACAGGCTGTTGACCATCAT	95
CYP2C19	TTGCTTCCTGATCAAAATGG	GTCTCTGTCCCAGCTCCAAG	108
CYP2C18	ATGAACAGTGCTCGGGACTT	TGGCTATCAAGCTTTCAACAG	100
CYP2D6	TGGACTTCCAGAACACACCA	CCCATTGAGCACGACCAC	104
CYP1A2	CTTCGTAAACCAGTGGCAGG	AGGGCTTGTTAATGGCAGTG	110
CYP2C8	CTCGGGACTTTATGGATTGC	CAGTGCCAACCAAGTTTTCA	93
CK7	CTGCCTACATGAGCAAGGTG	GGGACTGCAGCTCTGTCAAC	108
AQP1	CTCTCAGGCATCACCTCCTC	GGAGGGTCCCGATGATCT	109
CFTR	ACAGAAGCGTCATCAAAGCA	CCACTCAGTGTGATTCCACCT	100
SOX9	GACGCTGGGCAAGCTCT	GTAATCCGGGTGGTCCTTCT	106
HNF1α	CCTCAAAGAGCTGGAGAACCT	GACTTGACCATCTTCGCCAC	108
PROX1	ACAGGGCTCTGAACATGCAC	GGCATTGAAAAACTCCCGTA	101
TBX3	CTTCCACCTCCAGCAGCA	GCCATGTACGTGTAGGGGTA	90
CD13	AACCTCATCCAGGCAGTGAC	AAGCCTGTTTCCTCGTTGTC	92
CD133	CCATTGGCATTCTCTTTGAA	TTTGGATTCATATGCCTTCTGT	110
EpCAM	CTGAATTCTCAATGCAGGGTC	CCCATCTCCTTTATCTCAGCC	148
HHEX	CCTCTGTACCCCTTCCCG	GGGGCTCCAGAGTAGAGGTT	90
TGR5	CAGCAACTCCCTGACACTCA	TCTTGGTCCTGGGGACAG	110
HNF6	GGAGGATGTGGAAGTGGCT	TGTTGCCTCTATCCTTCCCA	108

Table S2. List of 1st and 2nd antibodies used for this study, related to Figures 2-4 and S2

Primary antibodies

	Species	Company (catalogue number)
AFP	Rabbit	Dako (A000829)
AFP	Mouse	Sigma-Aldrich (A8452)
ALB	Rabbit	Dako (A0001)
ALB	Mouse	Nippon bio-test laboratories (0902-1)
HNF4α	Goat	SantaCruz (sc-6556)
CK7	Mouse	Dako (M7018)
CD49f	Rat	BD Pharmingen (555734)
PKC	Rabbit	SantaCruz (sc-216)
CTNNB1	Mouse	BD Pharmingen (610153)
AQP1	Rabbit	SantaCruz (sc-20810)
OCT4	Rabbit	SantaCruz (sc-9081)
GATA4	Goat	SantaCruz (sc-1237)
FOXA2	Goat	SantaCruz (sc-6554)

Secondary antibodies

	Species	Company (catalogue number)
Alexa Fluor 488 anti-Rabbit IgG	Donkey	Life technologies (A21206)
Alexa Fluor 488 anti-Goat IgG	Donkey	Life technologies (A11055)
Alexa Fluor 555 anti-Goat IgG	Donkey	Life technologies (A21432)
Alexa Fluor 555 anti-Mouse IgG	Donkey	abcam (ab150110)
Alexa Fluor 647 anti-Goat IgG	Donkey	Life technologies (A21447)

Supplemental experimental procedures

Animals

C57BL/6 mice were used in the present study. All animal experiments were approved by the institutional Animal Care and Use Committee of the University of Tokyo.

Human primary hepatocyte culture

Human cryopreserved hepatocytes and all cell culture media were purchased from Biopredic International (Rennes, France). Hepatocytes were cultured according to the manufacturer's protocol. Briefly, hepatocytes were thawed using thawing medium and seeded at a density of 0.4 x 10^6 cells/well onto 24-well collagen I coated plates in seeding medium. After 1 day of culture, the medium was replaced with incubation medium and culture continued for 5 days.

Analysis of mouse fetal liver cells

Mouse fetal livers were collected from C57BL/6 mice at E14.5. The livers were minced and dissociated in Liver Digest Medium (Life technologies, California, US) for 15 min. The fetal liver cell suspension was passed through a 40 μm cell strainer (BD Biosciences, New Jersey, US) to obtain a single cell suspension. Then, cells were blocked by Fc block reagent and incubated with PE-conjugated anti-CPM antibody and FITC-conjugated anti-DLK1 antibody. PE and FITC-conjugated isotype controls were used as negative controls. CPM-positive (CPM^+) and -negative (CPM^-) cells were isolated by a MoFlo XDP cell sorter (Beckman Coulter, Inc, California, US).

Quantitative RT-PCR

Human fetal and adult liver RNAs were obtained from Gene Technology, Inc. (St. Louis, Missouri, US) and Life technologies, respectively.

Total RNA from cells was extracted with TRIzol reagent (Life technologies) according to the manufacturer's protocol. First-strand cDNA was synthesized using the PrimeScriptII 1st strand cDNA Synthesis Kit (Takara bio, Shiga, Japan). Quantitative RT-PCR was performed with the cDNA using specific primers for mouse and human genes. All data were calculated using the ddCt method with β-actin as a normalization control. Primers are listed in the Table S1.

Flow cytometric analysis for hiPSCs

Flow cytometric analysis was used to detect CPM^+ or $DLK1^+$ cells. Cells were dissociated using 0.05% trypsin/0.5 mM EDTA solution and then resuspended in PBS containing 0.03% BSA (PBS-BSA). Cells were incubated for 20 min with FcR blocking reagent (Miltenyi Biotech, Bergisch-Gladbach, Germany) followed by incubation with anti-Carboxypeptidase M antibody (Abcam, Massachusetts, US) or anti-DLK1 antibody (LivTech, Kanagawa, Japan) for 30 min on ice. Purified Mouse IgG1 isotype control (BioLegend, California, US) was used as negative control. Cells were washed and labeled with PE-conjugated anti-mouse IgG1 (BioLegend) for 30 min on ice.

Growth rate of CPM^+ cells

After the CPM^+ cells reached 50% confluence, they were passaged onto mitomycin C-treated MEF feeder cells. Cells were seeded into each well of a 12-well plate (Corning, New York, US) at 2.0 x 10^4 cells/cm^2. Cell proliferation was monitored in triplicate using a hemocytometer.

Immunohistochemistry and Immunocytochemistry

Fetal mouse was embedded in OCT compound (Sakura Finetek Japan., Co., Ltd., Tokyo, Japan). 10 μm sections were prepared and mounted on glass slides coated with APS (Matsunami glass Ind. Ltd., Osaka, Japan). Sections were fixed in 4% paraformaldehyde solution in PBS for 10 min, and washed three times with PBS. The sections were treated with 3% hydrogen peroxide (Wako Pure Chemical Industries, Ltd.) in methanol for 20 min. After washing three times with PBS, sections were blocked for 20 minutes with 4% skim milk in PBS and then incubated with anti-CPM antibody in a moisture chamber at 4°C overnight. They were again washed three times in PBS and incubated with biotinylated secondary antibody for 40 min at room temperature. Then, the sections were treated with an ABC-PO kit (Vector Laboratories Inc., California, US) for 1 hr at room temperature. Finally, the immunoreactive cells were visualized by 3,3'-diaminobenzidine tetra-hydrochloride (Dojin Laboratories, Kumamoto, Japan) and then counterstained with Hematoxylin (MERCK, Darmstadt, Germany) for 5 min.

Cultured cells were fixed in 10% buffered formalin solution (Wako Pure Chemical Industries, Ltd.) at room temperature for 10 minutes, and washed three times with PBS. Cells were then treated with PBS containing 0.2% Triton-X 100 (Wako Pure Chemical

Industries, Ltd.) at room temperature for 15 minutes. After washing three times with PBS, cells were blocked for 20 minutes with 4% skim milk in PBS and then incubated with primary antibodies at 4°C overnight. The cells were washed three times with PBS, incubated with appropriate fluorescein-conjugated secondary antibodies and then counterstained with Hoechst33342 (Sigma-Aldrich Corporation, St. Louis, US). Primary and secondary antibodies used for immunocytochemical analysis are shown in Table S2.

PAS staining

PAS staining was performed according to the standard protocol using Cold Schiff's Reagent (Wako Pure Chemical Industries, Ltd.).

CYP3A4 activity

CYP3A4 activity was determined by CYP3A4 P450-Glo assay with Luciferin-IPA (Promega, Wisconsin, USA), according to the manufacturer's protocol.

ALB and urea assay

ALB and urea levels in the cell culture supernatant were determined by ALB ELISA kit or Urea Assay Kit (Abcam) according to the manufacturer's protocol.

Acetylated Low Density Lipoprotein (Dil-Ac-LDL) labeling

Cells were incubated with 5 μg/ml Dil-Ac-LDL (AlfaAesar, Massachusetts, US) for 4 hrs at 37^{o}C. Then cells were washed with PBS and counterstained with Hoechst33342.

Data analysis

Data are expressed as mean ± SEM and analyzed by Student's t-test. The statistical significance was determined at $P<0.05$.