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Conversion of Human Hepatoma Cells by 520d-5p to Benign or Normal Liver Tissues via a stemness-Mediated Process

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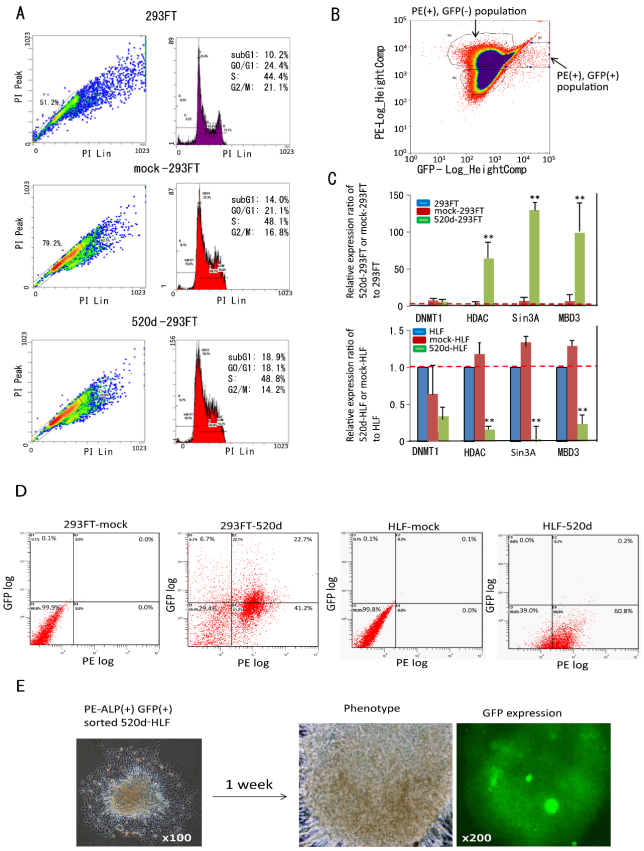
SUPPLEMENTARY



Supplementary Figure S1: A) DNA content in 293FT, mock-293FT and 520d-293FT were assessed in approximately 20,000 collected events. GFP-positive cells in mock-293FT and 520d-293FT were sorted. Cell cycle analysis of 520d-293FT showed increases and decreases in the S and G0 phases, respectively, with synchronized and

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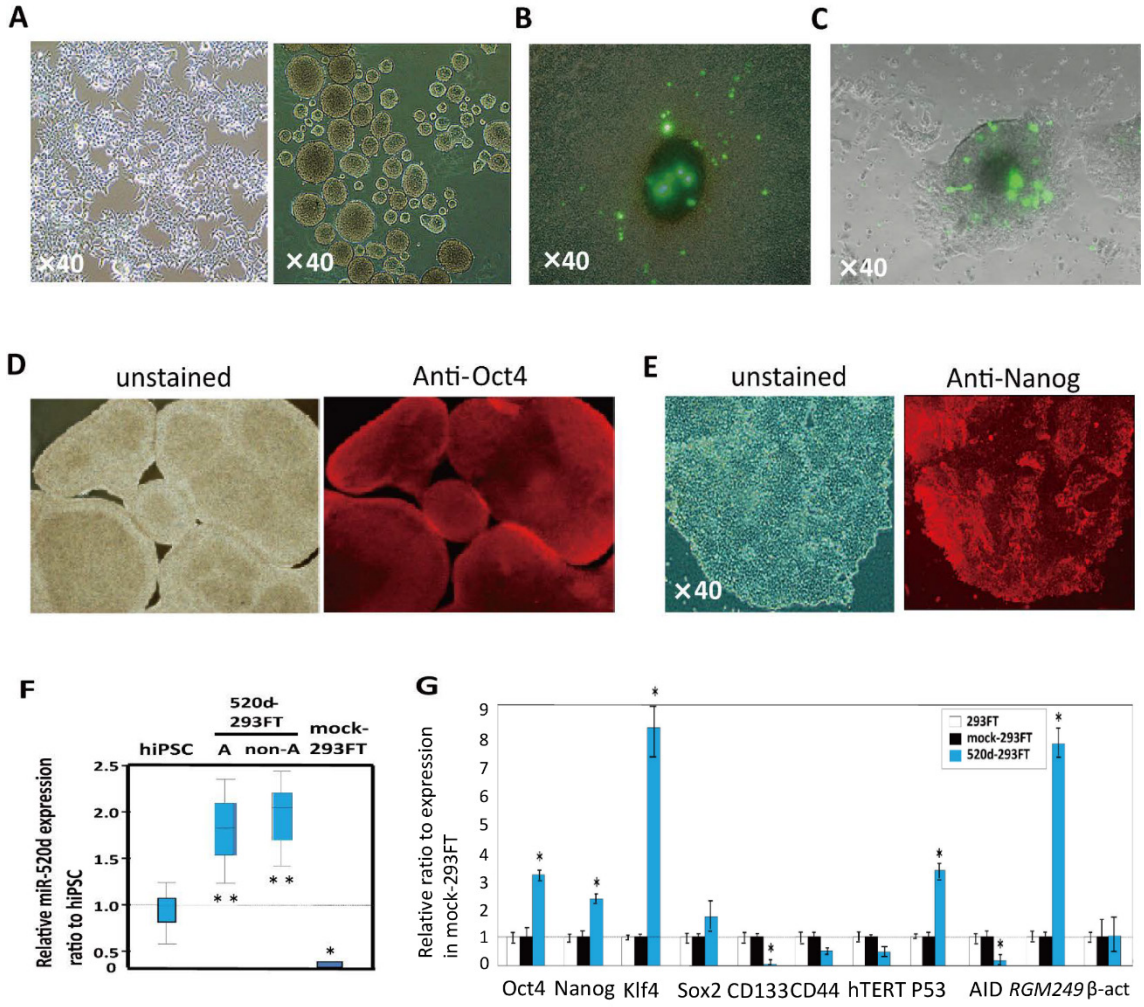
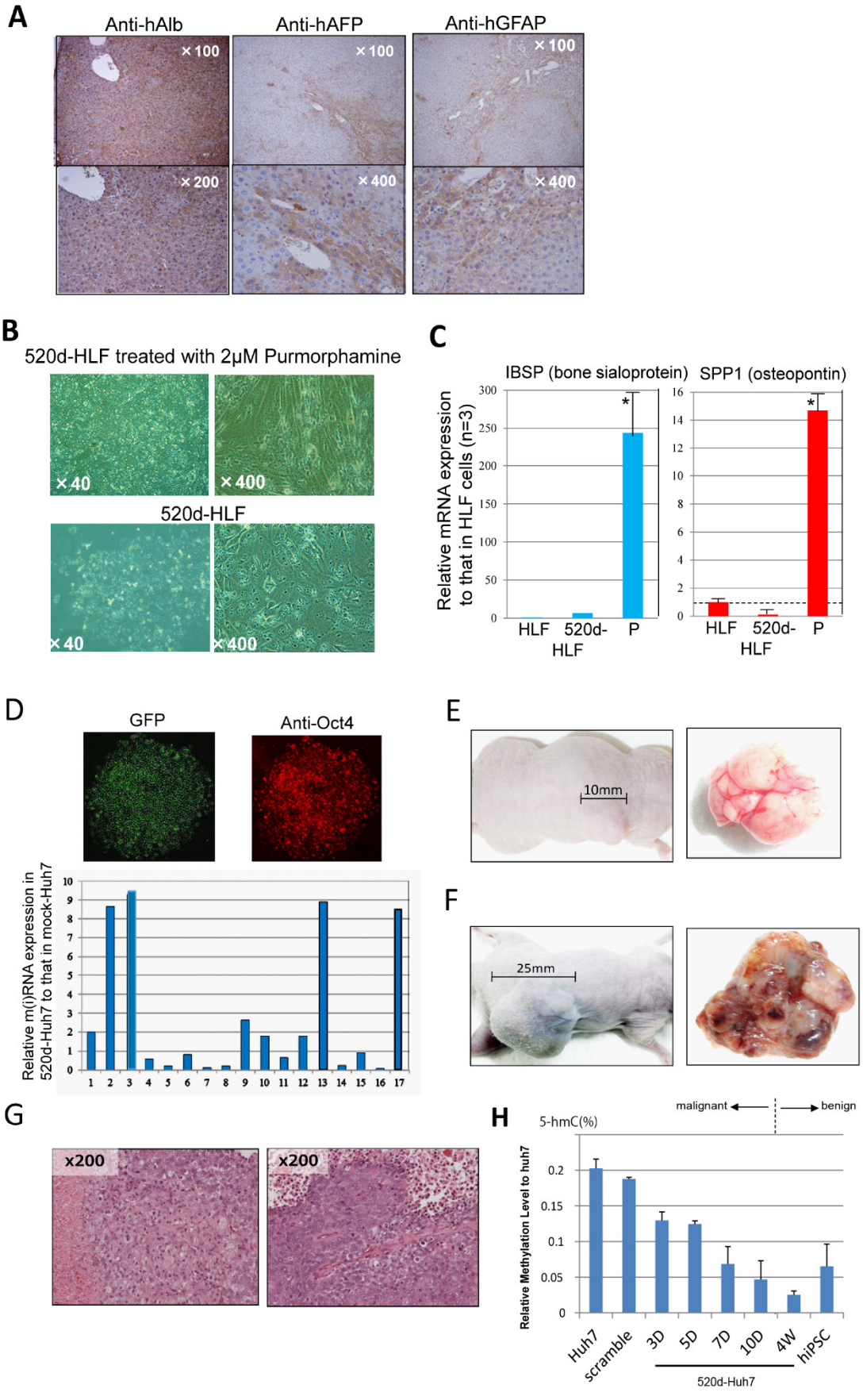
homogeneous proliferation compared with 293FT and mock-293FT, although the effect of miR-520p on G0, S-phase did not appear to be significant. B) Sorted immature populations were shown as PE positive cells or GFP (+) and ALP-PE (+) cells as arrows indicated. The cells were maintained in an immature state for two weeks after sorting. Although we found GFP (-) cells more than cells received 2% formaldehyde treatment due to the leakage of GFP during staining process, GFP (-) cells post-sorting had a similar populations to GFP (+) cells regarding gene expression and phenotype. C) Transcriptional examination of methylation status to determine the 520d-293FT reprogramming level. DNMT1 was not significantly expressed compared with mock-293FT, although HDAC, Sin3A and MBD3 expression levels were significantly upregulated (P<0.01) (top). In HLF, DNMT1 was not significantly expressed compared with mock-HLF, but HDAC, Sin3A and MBD3 levels were significantly downregulated (P < 0.01), unlike those in 293FT (bottom). Significant differences were not observed in expression levels between 293FT and HLF or between mock-293FT and mock-HLF, but theaverage relative ratio of 520d-293FT to 520d-HLF was 261.3 (range:11.9-2164.8). Data (n = 9) were analyzed with a Mann–Whitney U test. **: P < 0.01. D) FACS analysis in which mock-and 520d-293FT or mock- and 520d-HLF were compared. After 3 days, GFP positive or ALP positive cell frequencies were estimated. After one week under culture conditions to maintain an immature state, the majority of 520d-expressing 293FT and HLF cells expressed the pluripotent marker ALP (PE-labeled). E) GFP (+) and ALP-PE (+) cells were selected and maintained in an immature state for 2weeks after sorting. The phenotype of these cells before sorting was similar to that of iPS-like cells, and the sorted HLF continued to express GFP after sorting (left; two weeks post-sorting, right; three weekspost-sorting).

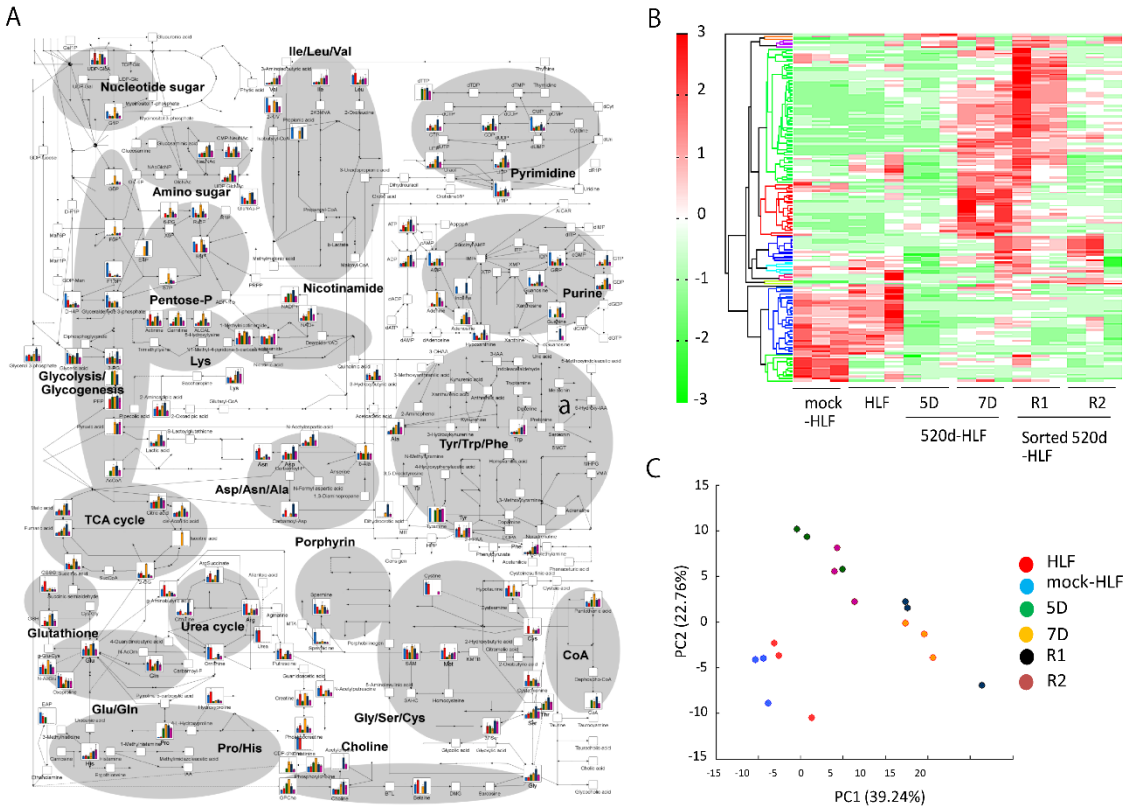


Supplementary Figure S2: Result of In vitro study and microscopic observations in miR-520d-virus-infected 293 FTcells (520d-293FT) were shown. A) Phenotypic changes in 520d-293FT were evaluated microscopically. Changes in cell morphology of 520d-293FT (right) was shown. Many non-adherent cells as well as adherent cells emerged after transfection in12-24 hours. 293FT cells (control) were shown (left). B) Confirmation of GFP expression in 520d-293FT that resembled a human-induced pluripotent stem cell. GFP-positive non-adherent cells were cultured in feeder cell-free ES cell medium. C) Time-lapse observations of an induced cell with GFP expression for 12 hours (x40 magnification) to show morphology and proliferation. Observation of another cell in video mode is provided as Supplementary video 7.520d-293FT maintained in Repro Stem medium grew up while maintaining the form of the colony unlike those cultured in DMEM. Scattered spheroid colonies were inter linked through long, branched groups of cells. D) Immunocytochemistry in a representative round cell with an anti-Oct4 antibody and a Rhodamine Red-conjugated secondary antibody. Oct 4 was strongly expressed. Unstained cells (left) and cells with Oct4 staining (right) were shown. E) Immunocytostaining with an anti-Nanog antibody concomitantly with GFP expression. Three days to one week later, the cells formed larger colonies and maintained a Nanog positive state under the same culture conditions. F) The effects on miRNA expression were confirmed in 520d-293FT and the relative ratio to hiPSCs was shown. 520d-293FT stably expressed miR-520d-5 pin both the adherent and non-adherent states in culture and the viral infection efficiency was greater than 99.1% according to GFP-positive cell sorting. miR-520d was significantly upregulated in 520d-293FT compared with hiPSCs. *:P<0.05,**:P<0.01. Data (n 18 = 9) were analyzed using a Mann–Whitney U test. A:adherent cells, non-A: non-adherent spherical cells. G) Relative ratio of the representative gene expression profile to that in mock-293FT by RT-PCR. Data depict the average relative ratio of 520d-293FT to mock-293FT, and a significant difference was shown, as*: P<0.01(n = 5). The pluripotent markers, P53 and RGM249 were upregulated, whereas AID was downregulated. Cancer stem cell (CSC) markers (CD133 and CD44) were not upregulated.



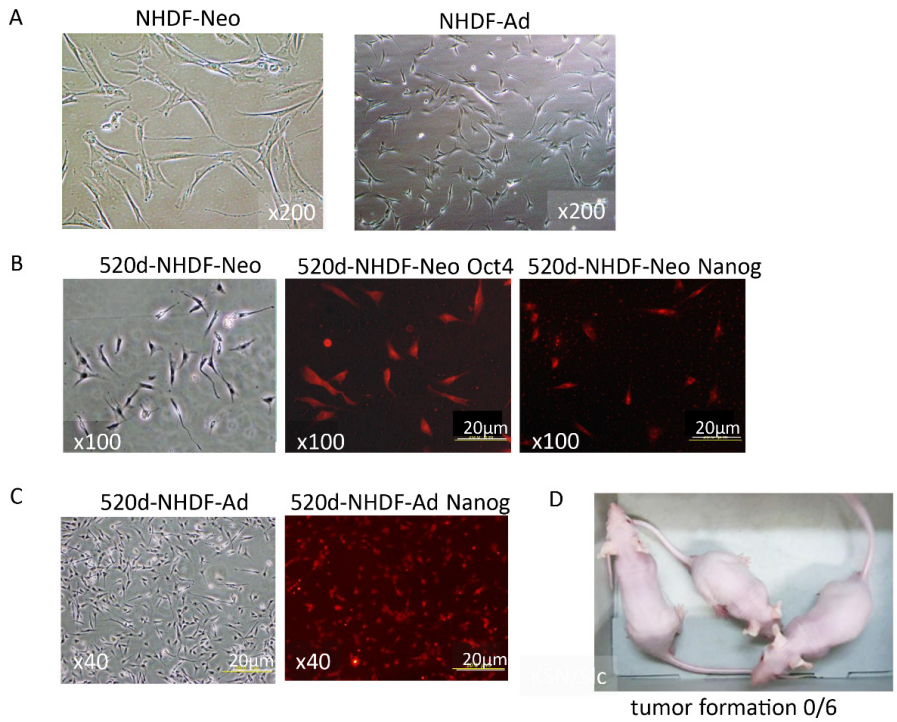
Supplementary Figure S3: A) Immunohistochemical analysis of liver tissue generated from 520d-HLF cells in xenograft model. (left) Human albumin was expressed strongly in hepatocytes from liver tissue generated from 520d-HLF cells in a xenograft model. (middle-right) Human AFP or GFAP were expressed weakly in the cytoplasm

of hepatocytes. B-C) Osteoblastic differentiation from 520d-HLF cells was induced morphologically and transcriptionally. B) Morphological changes were shown in 520d-HLF that were treated with 2μM purmorphamine (top) compared with untreated 520d-HLFcells (bottom). C) IBSP (bonesialoprotein) and SPP1(osteopontin) were strongly expressed in 520d-HLFcells treated with 2 μM purmorphamine (n = 4). P: purmorphamine-treated 520d-HLF, **:P<0.01. Tumorigenicity of well-differentiated hepatomacells (Huh7) received miR-520d-5p was examined. D) Induction of pluripotency by miR-520d in Huh7. Colonies of small round cells emerged within 12 days. Both GFP (lefttop) and Oct4 (right top) expression were confirmed by immunocytochemistry. Average geneexpression levels were examined (n= 5) and pluripotent marker gene and P53 mRNA levels were upregulated compared with mock-Huh7 (rightcolumn). Alb,c-Myc, AID and RGM249 levels were downregulated. 1-17:Oct4, Nanog, P53, hTERT, c-Myc, PROM1, CD44, AID, HDAC, DNMT1, Sin3A, MBD3, Lin28, RGM249, AFP, Alb and miR-520d. E) With the same viral vehicle titer used in the previous in vivo study with HLF, ten mice inoculated mock-Huh7 formed a tumor (top). The HE stain of representative tumor was shown. Well-differentiated neoplastic cells (partly squamous cell carcinoma-like cells) with substantial or alveolar arrangement were shown (bottom; 14x100). F) 520d-Huh7 was cultured for one week (once per week, we infected cells with the viral construct in vitro), tumorigenicity was confirmed one month after inoculation. G) Fifty percent of inoculated mice generated less-differentiated tumors one month later; the remaining mice did not generate tumors (n = 4). HE staining (x200 magnification) showed that the tumors were identical with low-differentiated hepatoma (left) and poorly-differentiated hepatoma(right). H) Average methylation rate of Huh7 was 0.20% and the data was standardized, compared with that in Huh7. An average hmC (%) in Huh7 cells was estimated to understand general methylation level during de-differentiation process by miR-520d-5p. Induction of hypermethylation was not induced in this cell type, but decreasing methylation level after 3D were observed, followed by the decrease less than iPS level a month later (n = 3).

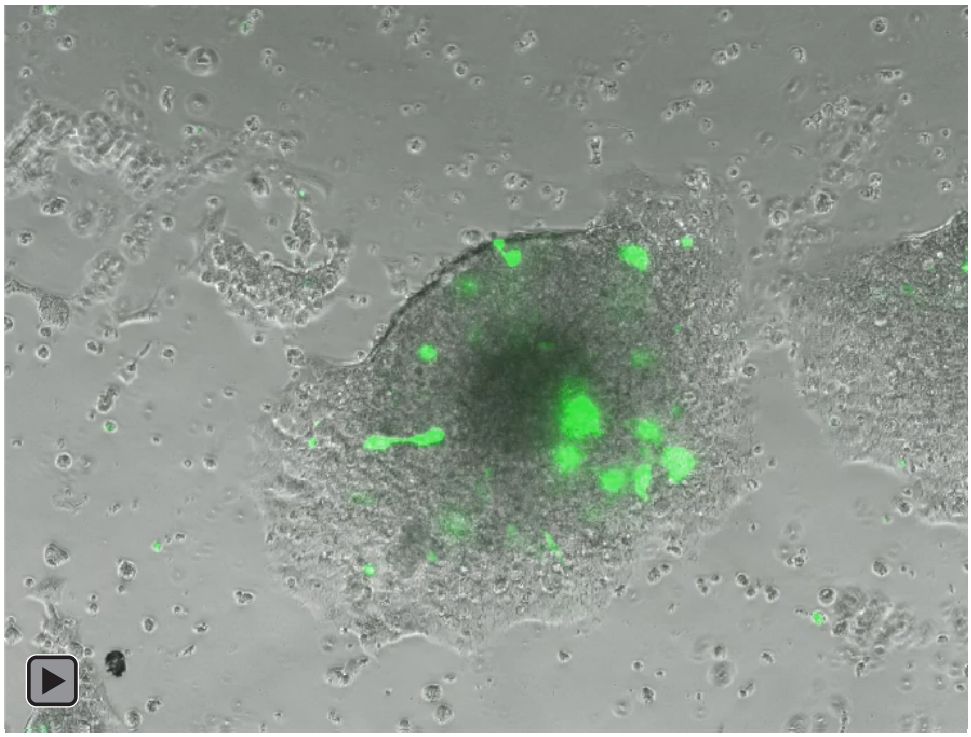


Supplementary FigureS4: A) Summarized pathway map (original summarized scheme) from HMT analysis. The result of this analysis was described in the discussion section in this text. B) heatmap (original data) obtained from metabolomic analysis. Mock-HLF and HLF (parental cells) were prominently different from the other four types of cells that expressed miR-520d-5p. C) A principal component analysis (PCA). Mock-HLF and HLF were found to have similar patterns, and the patterns of 7D and R1 were similar. R2 appears to possess similar characteristics to those of 5D and 7D (or R1).

SupplementaryFigureS5: The10predictedbindingsites(showninredletters)ofmiR-520d-5pinthe3'UTR 17 (1356-3805)ofELAVL2(1-3805)areshown.Twosites (1853-1880 and 2235-2249in 3'UTR)wereinvestigatedwithaluciferasereporterexpressionassay.Thesesites werepredictedbasedonthefourdatabasesdescribedabove.Bases 1607-1626 of the3'UTR wereused forasense primer sequence.



Supplementary Figure S6: Tumorigenicity of miR-520d- expressing fibroblasts (NHDF-Neo and-Ad) in KSN/Slc mice. A) Parental cells [NHDF-Neo (left) and-Ad (right), x200 magnification] were infected with a miR-520d-expressing lentiviral vector and inoculated into the right hindquarters of KSN/Slc mice. B) The fibroblast lines are represented as 520d-NHDF-Neo and 520d-NHDF-Ad. The phenotype of 520d-NHDF-Neo is shown (left; x100 magnification). Immunocytochemistry revealed the upregulation of Nanog (right) and Oct4 (middle) in 520d-NHDF-Neo (whitebar = 20μm). C) The phenotype of 520d-NHDF-Ad is shown (left; x40 magnification). Immunocytochemistry revealed the upregulation of Nanog expression (middle; white bar = 20μm). Average mRNA expression level of 520d-NHDF-Ad to mock-NHDF-Ad was shown. D) The tumorigenicity of 520d-induced fibroblasts was examined in KSN/Slc mice. Neither 520d-NHDF-Neo (n = 3) nor -Ad (n = 3; right) generated tumors in mice.



Supplementary File (video) 7: 520d-HLF cells in video mode is provided bytime-lapseimage.

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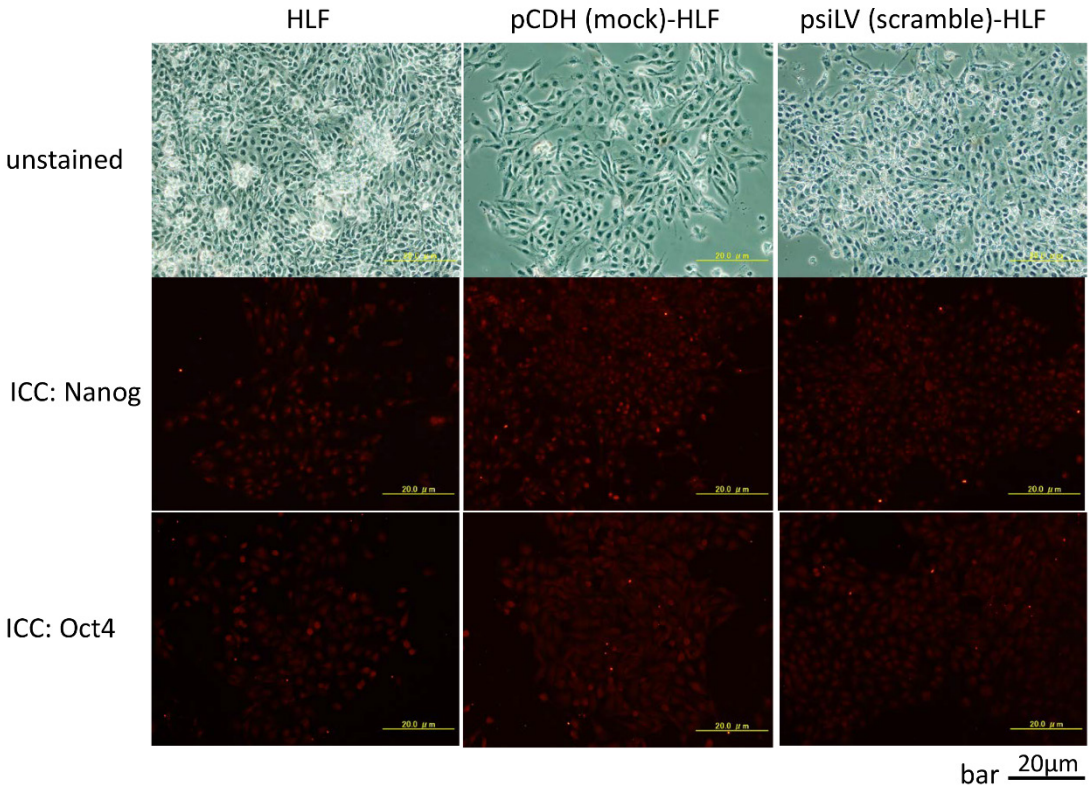
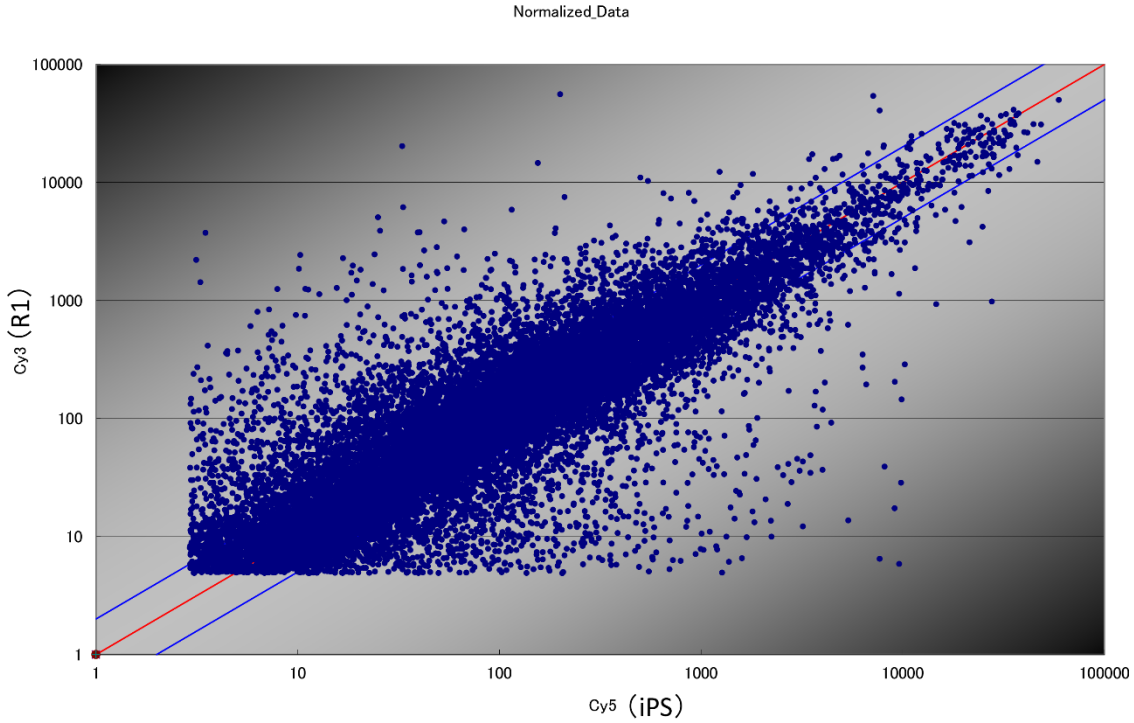


Supplementary File 8: Oct4 or Nanog expression in a parental HLF, pCDH-HLF or psiLV (scrambled)-HLF was shown. pCDH and psiLV were used as controls for pMIR-520d-5p and siELAVL2 [unstained (top), ICC: Nanog (middle) and ICC:Oct4 (bottom)]. Nanog and Oct4 were both weakly expressing the pluripotent markers. Approximately 8% of populations in HLFcells seemed to be slightly expressing both markers stronger than other populations around them. Also, Oct4 was expressed in each sample weaker than Nanog. ICC: immunocytochemistry.



Supplementary File 9: Normalized data between R1 and hiPSC in microarray analysis is shown as a graph.

symbol	description	LOG2[ratio(R1/iPS)]
SEMA3C	Semaphorin-3C Precursor (Semaphorin-E)(Sema E)	10.06
MAGEC2	Melanoma-associated antigen C2 (MAGE-C2 antigen)(MAGE-E1 antigen)(Hepatocellular carcinoma-associated antigen 587)(Cancer/testis antigen 10)(CT10)	9.45
TM4SF1	Transmembrane 4 L6 family member 1 (Tumor-associated antigen L6)(Membrane component surface marker 1)(M3S1)	9.26
RP13-36C9.3	Cancer/testis antigen 45-3 (CT45-3)	8.75
IGFBP7	Insulin-like growth factor-binding protein 7 Precursor (IGF-binding protein 7)(IGFBP-7)(IBP-7)(MAC25 protein)(Prostacyclin-stimulating factor)(PGI2-stimulating factor)(IGFBP-rP1)	8.12
NT5E	5'-nucleotidase Precursor (EC 3.1.3.5)(Ecto-5'-nucleotidase)(5'-NT)(CD73 antigen)	7.87
MMP1	Interstitial collagenase Precursor (EC 3.4.24.7)(Matrix metalloproteinase-1)(MMP-1)(Fibroblast collagenase) [Contains 22 kDa interstitial collagenase;27 kDa interstitial collagenase]	7.65
PAGE2	G antigen family E member 2 (Prostate-associated gene 2 protein)(PAGE-2)	7.52
FILIP1	Filamin-A-interacting protein 1 (FILIP)	7.51
ALPK2	Alpha-protein kinase 2 (EC 2.7.11.-)(Heart alpha-protein kinase)	7.29
CCL2	C-C motif chemokine 2 Precursor (Small-inducible cytokine A2)(Monocyte chemoattractant protein 1)(Monocyte chemotactic protein 1)(MCP-1)(Monocyte chemotactic and activating factor)(MCAF)(Monocyte secretory protein JE)(HC11)	7.24
PRAME	Melanoma antigen preferentially expressed in tumors (Preferentially expressed antigen of melanoma)(OPA-interacting protein 4)(OIP4)	7.08
IL18	Interleukin-18 Precursor (IL-18)(Interferon-gamma-inducing factor)(IFN-gamma-inducing factor)(Interleukin-1 gamma)(IL-1 gamma)(Iboctadekin)	6.98
AC069282.6	Putative uncharacterized protein FLJ21075 Precursor	6.85
FGB	Fibrinogen beta chain Precursor [Contains Fibrinopeptide B]	6.84
IFI44	Interferon-induced protein 44 (p44)(Microtubule-associated protein 44)	6.84
MVP	Major vault protein (MVP)(Lung resistance-related protein)	6.76
SPANXD	Sperm protein associated with the nucleus on the X chromosome D (SPANX-D)(SPANX family member D)(Nuclear-associated protein SPAN-Xd)(Cancer/testis antigen 11.4)(CT11.4)	6.71
IFI44L	Interferon-induced protein 44-like	6.69
CXCL2	C-X-C motif chemokine 2 Precursor (Macrophage inflammatory protein 2-alpha)(MIP2-alpha)(Growth-regulated protein beta)(Gro-beta) [Contains GRO-beta(5-73)(GRO-beta-T)(SB-251353)(Hematopoietic synergistic factor)(HSF)]	6.66

Supplementary File 10: Upregulated 20 genes (more than 8 fold) were representatively shown between R1 and hiPSC for reference (n = 1). R1 was a cell population formed liver tissue in vivo.

symbol	description	LOG2[ratio(Cy3/Cy5)]
LIN28	Lin-28 homolog A (Zinc finger CCHC domain-containing protein 1)	-10.69
TACSTD1	Tumor-associated calcium signal transducer 1 Precursor (Major gastrointestinal tumor-associated protein GA733-2)(Epithelial cell surface antigen)(Epithelial glycoprotein)(EGP)(Adenocarcinoma-associated antigen)(KSA)(KS 1/4 antigen)(Cell surface glycoprotein Trop-1)(CD326 antigen)	-10.22
EPCAM	Tumor-associated calcium signal transducer 1 Precursor (Major gastrointestinal tumor-associated protein GA733-2)(Epithelial cell surface antigen)(Epithelial glycoprotein)(EGP)(Adenocarcinoma-associated antigen)(KSA)(KS 1/4 antigen)(Cell surface glycoprote	-9.05
GAL	Galanin Precursor [Contains Galanin;Galanin message-associated peptide(GMAP)]	-8.62
L1TD1	LINE-1 type transposase domain-containing protein 1 (ES cell-associated protein 11)	-8.44
SERPINB9	Serpin B9 (Cytoplasmic antiproteinase 3)(CAP-3)(CAP3)(Proteinase inhibitor 9)	-8.05
FGF13	Fibroblast growth factor 13 (FGF-13)(Fibroblast growth factor homologous factor 2)(FHF-2)	-8.02
SLC16A9	Monocarboxylate transporter 9 (MCT 9)(Solute carrier family 16 member 9)	-7.94
SLC7A3	Cationic amino acid transporter 3 (CAT-3)(Cationic amino acid transporter y+)(Solute carrier family 7 member 3)	-7.83
SALL4	Sal-like protein 4 (Zinc finger protein SALL4)	-7.82
TDGF1	Teratocarcinoma-derived growth factor 1 Precursor (Epidermal growth factor-like cripto protein CR1)(Cripto-1 growth factor)(CRGF)	-7.71
CRABP1	Cellular retinoic acid-binding protein 1 (Cellular retinoic acid-binding protein I)(CRABP-I)	-7.42
LECT1	Chondromodulin-1 Precursor (Chondromodulin-I)(ChM-I)(Leukocyte cell-derived chemotaxin 1) [Contains Chondrosurfactant protein(CH-SP)]	-7.36
ZSCAN10	Zinc finger and SCAN domain-containing protein 10 (Zinc finger protein 206)	-7.28
ZFP42	Zinc finger protein 42 homolog (Zfp-42)(Reduced expression protein 1)(REX-1)(hREX-1)(Zinc finger protein 754)	-7.18
EDNRB	Endothelin B receptor Precursor (ET-B)(Endothelin receptor non-selective type)	-7.17
FOXN3	Forkhead box protein N3 (Checkpoint suppressor 1)	-7.13
GPM6B	Neuronal membrane glycoprotein M6-b (M6b)	-7.08
RASL11B	Ras-like protein family member 11B	-7.07
PXDN	Peroxidasin homolog Precursor (EC 1.11.1.7)(Vascular peroxidase 1)(Melanoma-associated antigen MG50)(p53-responsive gene 2 protein)	-7.06

Supplementary File 11: Downregulated 20 genes (more than 8 fold) were representatively shown between R1 and hiPSC for reference (n = 1).