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 the average 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-5p 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. 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, ast*: 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.


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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 2 μM purmorphamine (top) compared with untreated 520d-HLF cells (bottom). C) IBSP (bonesialoprotein) and SPP1 (osteopontin) were strongly expressed in 520d-HLF cells treated with 2 μM purmorphamine (n = 4). P: purmorphamine-treated 520d-HLF, **P<0.01. Tumorigenicity of well-differentiated hepatocellular carcinomas (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 gene expression levels were examined (n=5) and pluripotent marker gene and PS3 mRNA levels were upregulated compared with mock-Huh7 (right column). 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 Figure S4: 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).
Supplementary Figure S5: The 10 predicted binding sites (shown in red letters) of miR-520d in the 3' UTR of ELAVL2 are shown. Two sites (1853-1880 and 2235-2249) were investigated with a luciferase reporter expression assay. These sites were predicted based on the four databases described above. Bases 1607-1626 of the 3' UTR were used for a sense 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 (white bar = 20 μm). C) The phenotype of 520d-NHDF-Ad is shown (left; x40 magnification). Immunocytochemistry revealed the upregulation of Nanog expression (middle; white bar = 20 μm). 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.


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

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.

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