Supporting Information

Kajiwara et al. 10.1073/pnas.1209979109

SI Materials and Methods

Hepatic Differentiation in Vitro. For the hepatic differentiation of human induced pluripotent stem/embryonic stem cells (hiPS/ESCs), we initially applied a previously reported hepatic differentiation protocol designed for hESCs (1) with some modifications. Briefly, small clumps of undifferentiated hiPS/ESCs were seeded on Matrigel (growth-factor reduced; BD Biosciences)-coated plates and cultured in mouse embryonic fibroblast (MEF)-conditioned Primate ES cell medium supplemented with 4 ng/mL bFGF. When the hiPS/ESCs reached nearly 70% confluence, the medium was replaced with RPMI1640 (Nacalai Tesque) containing 1× B27 supplement (Invitrogen), 100 ng/mL activin A (PeproTech), 50 ng/mL Wnt3a (R&D Systems), and 1 mM sodium butyrate (NaB) (Sigma) for 1 d. On the following 2 d, sodium butyrate was omitted from the medium. After 3 d of culture in serum-free activin A-based medium, the culture medium was replaced with knockout-DMEM (KO-DMEM) containing 20% (vol/vol) knockout serum replacement (KSR), 1 mM L-glutamine, 1% (vol/vol) nonessential amino acids, 0.1 mM 2-mercaptoethanol (all from Invitrogen), and 1% (vol/vol) DMSO (Sigma) (differentiation medium) for 7 d. Finally, the cells were cultured in hepatocyte culture medium (Lonza) supplemented with 20 ng/mL hepatocyte growth factor (HGF) (PeproTech) and 20 ng/mL oncostatin M (OSM) (PeproTech) (maturation medium) for another 7 d. The medium was changed daily during the differentiation period.

For endodermal cell induction from single hiPS/ESCs, the hiPS/ESCs were incubated with Accutase (Innovative Cell Technologies) for 20 min and dissociated into single cells by pipetting. Cells were resuspended with RPMI1640 medium containing 1× B27 supplement, 100 ng/mL activin A, 50 ng/mL Wnt3a, and 10 μM Y27632 (Wako) and were seeded on Matrigel-coated culture dishes at a density of 1×10^5 cells/cm^2. Beginning the next day, Y27632 was omitted from the medium, and 0.5 mM NaB was added in the culture medium as described in the main text. For hepatic differentiation, further cultivation was performed with the differentiation medium and maturation medium described above from day 7 to day 13 and from day 14 to day 20, respectively.

Immunohistochemical Staining. The cells were fixed with PBS containing 4% (wt/vol) paraformaldehyde (PFA) for 10 min at room temperature. After washing the cells with PBS, nonspecific binding was blocked with PBS containing 5% (vol/vol) normal goat or donkey serum (Chemicon), 1% BSA (wt/vol) (Nacalai Tesque), and 0.1% (vol/vol) Triton X-100 for 45 min at room temperature. The primary antibodies used included HNF4A (1:500; Santa Cruz sc-6556), AFP (1:200; DAKO A0008), ALBUMIN (1:200; Bethyl A80-229A), A1AT (1:50; Invitrogen 180002), SOX17 (1:300; R&D Systems AF1924), and OCT3/4 (1:100; Santa Cruz sc-5279). The secondary antibodies used were cyanine3 (Cy3)-conjugated anti-goat IgG (1:500; Chemicon) for HNF4A, Alexa488-conjugated anti-rabbit IgG (1:500; Invitrogen) for AFP, Alexa488-conjugated anti-goat IgG (1:500; Invitrogen) for ALBUMIN and SOX17, Cy3-conjugated anti-rabbit IgG (1:500; Chemicon) for A1AT, and Cy3-conjugated anti-mouse IgG (1:500; Chemicon) for OCT3/4. Nuclei were stained with 1 μg/mL Hoechst 33342 (Invitrogen).

Flow Cytometric Analysis. To analyze the populations for albumin-positive cells, hepatic differentiated hiPS/ESCs were dissociated in 0.25% (wt/vol) Trypsin-EDTA (Invitrogen) and then resuspended in 2% FBS/PBS (vol/vol). The collected cell suspensions were fixed with PBS containing 4% PFA (wt/vol), permeabilized with 0.1% (vol/vol) Triton X-100, and stained with an antibody against albumin and an Alexa488-conjugated secondary antibody described above. To analyze the CXCR4-positive cells, endodermally differentiated hiPS/ESCs were dissociated in Accutase and stained with a PE-conjugated CXCR4 antibody (R&D Systems FAB170P). The analyses were performed using a FACS Aria II flow cytometer (BD Biosciences).

Functional Analysis of Hepatic Differentiated hiPS/ESCs in Vitro. To evaluate the glycogen production and storage of the hepatic differentiated hiPS/ESCs, Periodic acid-Shiff (PAS) staining was performed. The cultured cells were fixed in 3.3% (vol/vol) formalin for 10 min, and intracellular glycogen was stained using a PAS staining solution (Muto Pure Chemicals), according to the manufacturer's instructions. For the albumin secretion assay, the culture media of differentiated cells after a 24-h incubation were collected and evaluated using the Human Albumin ELISA Quantitation kit (Bethyl Laboratories) according to the manufacturer's protocol. To examine their ability to metabolize ammonia, the cells were cultured for 24 h in phenol red-free DMEM (Nacalai Tesque) supplemented with 1.5 mM ammonium chloride (Nacalai Tesque). The ammonia concentrations in the culture media were measured using an Ammonia-Test kit (Wako), according to the manufacturer's protocol. To evaluate the cytochrome P450 3A4 activity, a P450-Glo CYP3A4 Assay kit (Luciferin-IPA; Promega) was used according to the manufacturer's protocol. Readout was performed with the Centro LB 960 detection system (Berthold). The CYP450 activity was expressed as the relative light units per 50 μL of medium.

DNA Microarray Analysis. Total RNA was purified with a miRNeasy mini kit (QIAGEN) according to the manufacturer's protocol. DNA microarray analysis was performed as described previously (2), using a SurePrint G3 Human Gene Expression 8 × 60K kit (G4851A; Agilent) and a G2565CA Microarray Scanner System (Agilent). The data were analyzed using the GeneSpring GX 11 software package (Agilent). Reported microarray data have been deposited in the public database Gene Expression Omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession no. GSE38155.

Statistical Analysis. All statistical analyses were performed with a one-way repeated-measures ANOVA and the Bonferroni post hoc test.

1. Hay DC, et al. (2008) Efficient differentiation of hepatocytes from human embryonic stem cells exhibiting markers recapitulating liver development in vivo. *Stem Cells* 26:894–902.
2. Takahashi K, et al. (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. *Cell* 131:861–872.

Fig. S1. Characterization of hepatic differentiated hiPSCs (201B6) and hESCs (KhES3). (*A*) RT-PCR analysis of the expression of various CYP450 enzymes, ABC transporters, and enzymes involved in glucuronidation in the hepatic differentiated hiPS/ESCs on day 17. (*B*) Periodic acid-Schiff (PAS) staining was performed to detect intracellular glycogen. (Scale bar: 100 μm.) (*C*) Liver-specific cytochrome P450 (CYP3A4) metabolic activity of the undifferentiated (day 0) and hepatic differentiated hiPS/ESCs on day 17. Error bars indicate the SD (n = 3).

Fig. S2. Time-course images of the endodermal and hepatic differentiation from single hiPSCs. Shown are phase-contrast images of the endodermal differentiation from single hiPSCs (201B6 and 201B7) and the hepatic differentiation with further cultivation. A total of 0.5 mM NaB was added for 3 d (from day 1 to day 3). (Scale bar: 100 μm.) On day 21 of the hepatic differentiation, albumin staining was performed.

Fig. S3. Microarray analysis of sibling hiPSC lines. Microarray-based gene expression analysis showed that the global gene expression pattern, including 10 liver-related transcription factors (orange dots and letters), was similar between 201B6 and 201B7 hiPSC lines both in the undifferentiated state (*A*) and in CXCR4-positive cells on day 7 (*B*). Only the detectable probes are shown. The green lines indicate the diagonal and twofold changes between the two samples.

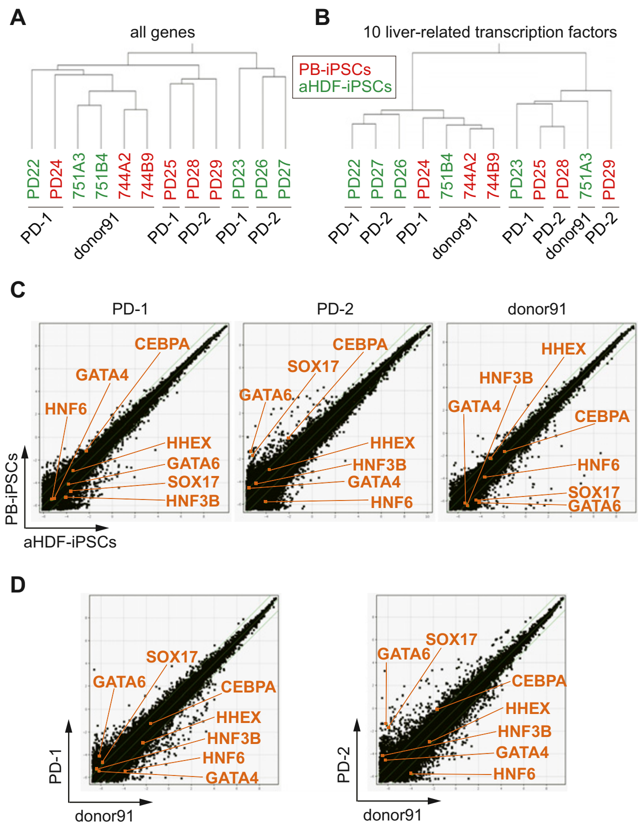

Fig. S4. Microarray analysis of hiPSC lines generated from the same individuals. (*A* and *B*) Hierarchical clustering analysis of undifferentiated peripheral blood (PB)-iPSCs and adult human dermal fibroblasts (aHDF)-iPSCs derived from the same individuals [Parkinson disease (PD) 1, PD-2, and donor91] was performed using all gene sets (*A*) and 10 liver-related transcription factors (*B*). (*C* and *D*) Scatter-plot analyses of undifferentiated PB-iPSCs and aHDF-iPSCs derived from the same individuals (PD-1, PD-2, and donor91) are shown. (*C*) Intraindividual comparisons between PB-iPSCs and aHDF-iPSCs. (*D*) Interindividual comparisons of PB-iPSCs. Ten liver-related transcription factors are indicated by orange dots and letters. Only the detectable probes are shown. The green lines indicate the diagonal and twofold changes between the two samples.

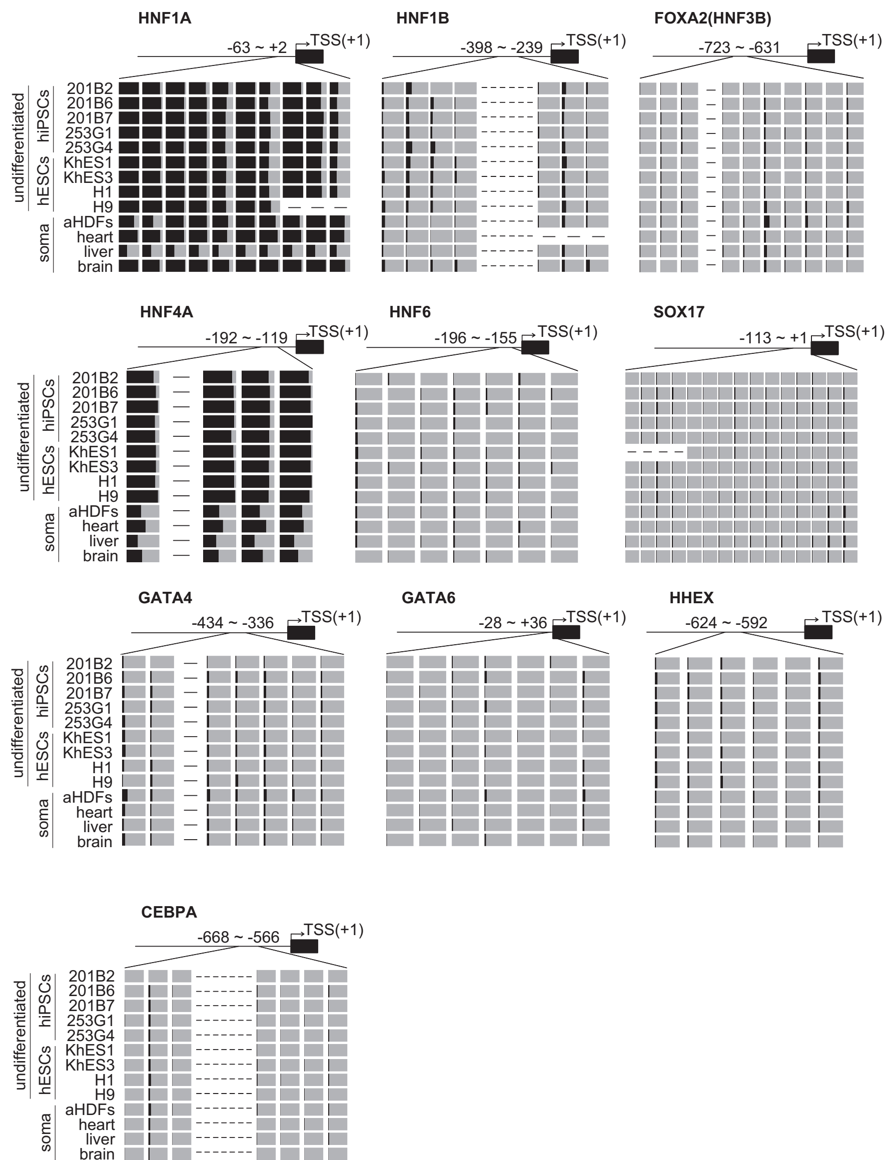

Fig. S5. Pyrosequencing analysis of the promoter of liver-related transcription factors in undifferentiated hiPS/ESC lines and somatic tissues. Genomic DNA was obtained from undifferentiated hiPSCs (201B2, 201B6, 201B7, 253G1, and 253G4) and hESCs (KhES1, KhES3, H1, and H9). Genomic DNAs of adult human dermal fibroblasts (aHDFs), heart, liver, and brain were used as controls. A single column represents one CpG site. Solid bars indicate the percentage of methylated CpG sites and shaded bars indicate the percentage of unmethylated CpG sites. A hyphen represents one CpG site that was not analyzed. TSS: transcription start site.

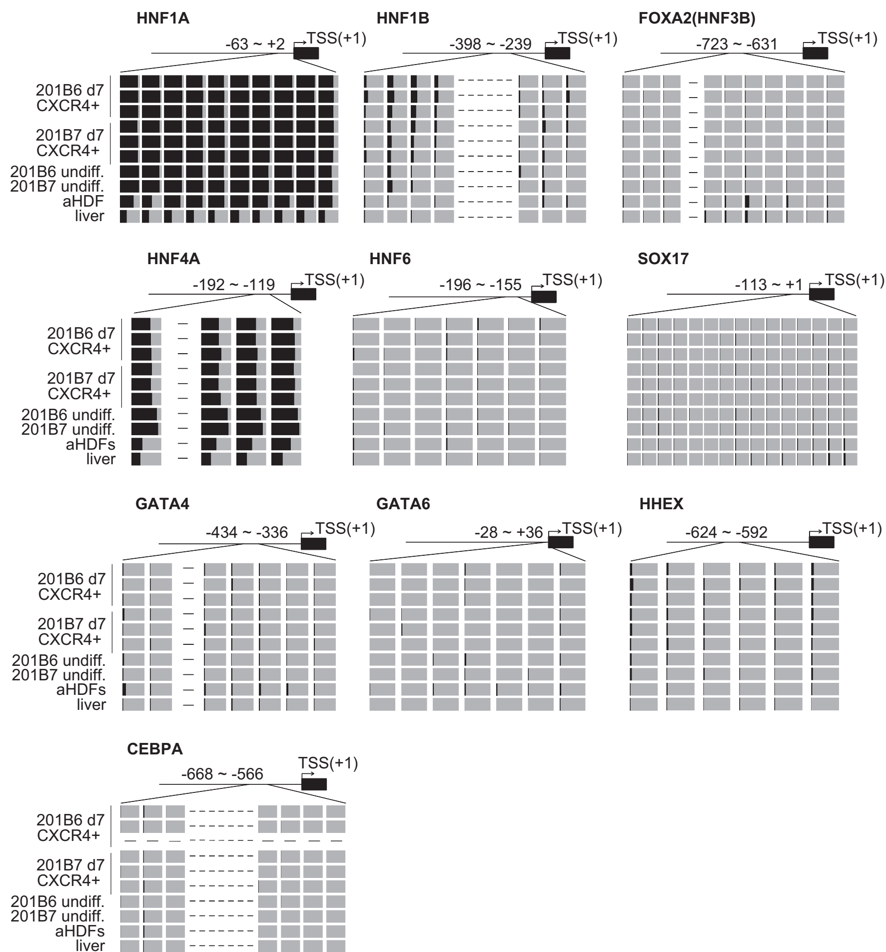

Fig. S6. Pyrosequencing analysis of the promoter of liver-related transcription factors in CXCR4-positive cells on day 7 of 201B6 and 201B7 hiPSC lines. Genomic DNA was obtained from CXCR4-positive cells on day 7 of 201B6 and 201B7 hiPSC lines, which were sorted by flow cytometry. Genomic DNAs of undifferentiated hiPSCs (201B6 and 201B7), adult human dermal fibroblasts (aHDFs), and adult liver were used as controls. A single column represents one CpG site. Solid bars indicate the percentage of methylated CpG sites and shaded bars indicate the percentage of unmethylated CpG sites. A hyphen represents one CpG site that was not analyzed. TSS: transcription start site.

Table S1. Cell information for the experiment

	Name	Vector	Factors	Origin	Passage no. in Figs. 4 and 5
aHDF-iPSCs	201B2	Retrovirus	O, S, K, c-MYC	aHDFs (Lot 1388)	21, 22, 23
	201B6				23, 24, 25
	201B7				23, 24, 25
	253G1		O, S, K		21, 22, 23
	253G4				23, 24, 25
	409B2	Episomal	O, S, K, L-MYC, LIN28, sh-p53		29, 30, 31
	414C2				28, 30, 31
Dental pulp (DP)-iPSCs	451F3	Episomal	O, S, K, L-MYC, LIN28, sh-p53	DP74 (HLA homozygous)	24, 25, 26
	454E2				23, 24, 25
	457C1				26, 27, 28
Peripheral blood (PB)-iPSCs	585A1	Episomal	O, S, K, L-MYC, LIN28, sh-p53	T lymphocytes (donor x)	23, 24, 25
	585B1				23, 24, 25
	604A1			T lymphocytes (donor y)	29, 30, 31
	604A3				29, 30, 31
	604B1				27, 28, 29
	622E1	Sendai virus	O, S, K, c-MYC		22, 23, 24
	622G1				22, 23, 24
	703A1			T lymphocytes (donor x)	21, 22, 23
	703B1				21, 22, 23
Cord blood (CB)-iPSCs	606A1	Episomal	O, S, K, L-MYC,LIN28, sh-p53	$CD34^+$ (donor a)	29, 30, 31
	606B1				27, 28, 29
	610A2			$CD34^+$ (donor b)	25, 26, 27
	610B1				24, 25, 26
	665A1	Sendai virus	O, S, K, c-MYC		23, 24, 25
	665A7				23, 24, 25
	TkCBV4-2	Retrovirus	O, S, K, c-MYC	$CD34^+/CD45^+$	60, 61, 62
	TkCBV5-6			$CD34^-/CD45^+$	55, 56, 57
	TkCB7-2			$CD34^+/CD45^+$	22, 23, 24
hESCs	KhES1	—	—	—	25, 26, 27
	KhES3				29, 30, 31
	H1				51, 52, 53
	H9				42, 43, 44
	ES03				72, 73, 74
	ES04				81, 82, 83
	ES06				50, 51, 52
aHDF- and PB-iPSCs from the same individuals					
aHDF-iPSCs	CiRA-PD22	Episomal	O, S, K, L-MYC, LIN28, sh-p53	Parkinson disease patient (PD-1)	15, 16, 17
	CiRA-PD23				15, 16, 17
PB-iPSCs	CiRA-PD24				21, 22, 23
	CiRA-PD25				27, 28, 29
aHDF-iPSCs	CiRA-PD26			Parkinson disease patient (PD-2)	21, 22, 23
	CiRA-PD27				23, 24, 25
PB-iPSCs	CiRA-PD28				17, 18, 19
	CiRA-PD29				27, 28, 29
aHDF-iPSCs	751A3			Adult healthy donor (donor91)	19, 20, 21
	751B4				19, 20, 21
PB-iPSCs	744A2				19, 20, 21
	744B9				19, 20, 21

K, KLF4; O, OCT3/4; S, SOX2; sh-p53, p53-shRNA.

Table S2. Primer information for RT-PCR

Target gene	RT-PCR primers	Sequence (5′–3′)
CYP1A1	hCYP1A1_F	ACC TGA ATG AGA AGT TCT ACA GC
	hCYP1A1_R	CTG GGG TTC ATC ACC AAA TAC A
CYP2C9	hCYP2C9_F	CCC TGG ATC CAG ATC TGC AA
	hCYP2C9_R	TGC TTG TCG TCT CTG TCC CA
CYP2C19	hCYP2C19_F	GGT GCT GCA TGG ATA TGA AGT G
	hCYP2C19_R	TGG ATC CAG GGG GTG CTT AC
CYP2D6	hCYP2D6_F	CCT GCT CAT GAT CCT ACA TCC
	hCYP2D6_R	ACC AGG AAA GCA AAG ACA CCA T
CYP3A4	hCYP3A4_F	CCA AGC TAT GCT CTT CAC CG
	hCYP3A4_R	TCA GGC TCC ACT TAC GGT GC
CYP7A1	hCYP7A1-RT-S	GTG CCA ATC CTC TTG AGT TCC
	hCYP7A1-RT-AS	ACT CGG TAG CAG AAA GAA TAC ATC
MRP2(ABCC2)	hMRP2_F	ACC TCC AAC AGG TGG CTT GCA
	hMRP2_R	ACA CCA ATC TTC TCC ATG CTA CC
MDR/TAP(ABCB11)	hMDRTAP_F	GTG CTG AGT AAG ATT CAG CAT GGG
	hMDRTAP_R	AGC ATG TCA TCT TCA GTT GCA TCC T
UGT1A1	hUGT1A1_F	GTG CCT TTA TCA CCC ATG CT
	hUGT1A1_R	TCT TGG ATT TGT GGG CTT TC
GAPDH	GAPDH-F	ACC ACA GTC CAT GCC ATC AC
	GAPDH-R	TCC ACC ACC CTG TTG CTG TA

Table S3. Primer information for real-time PCR

Target gene	Real-time PCR primers	Sequence (5′–3′)
OCT3/4(endo_3′-UTR)	hOct4-S1165	GAC AGG GGG AGG GGA GGA GCT AGG
	hOct4-AS1283	CTT CCC TCC AAC CAG TTG CCC CAA AC
A1AT	hAAT-RT-S	ACA TTT ACC CAA ACT GTC CAT T
	hAAT-RT-AS	GCT TCA GTC CCT TTC TCG TC
AFP	hAFP-qS	AAA TGC GTT TCT CGT TGC TT
	hAFP-qAS	GCC ACA GGC CAA TAG TTT GT
ALBUMIN	hALBUMIN-qS	CTT CCT GGG CAT GTT TTT GT
	hALBUMIN-qAS	TGG CAT AGC ATT CAT GAG GA
TDO2	h_TDO2_RT-s	GAC GGC TGT CAT ACA GAG CA
	h_TDO2_RT-as	CGC AGG TAG TGA TAG CCT GA
ASGR1	hASGR1-qS	CAC GTG AAG CAG TTC GTG TC
	hASGR1-qAS	CGG AGC GAG AGA ACC AGT AG
HNF4A	hHNF4α-RT-S	CCA CGG GCA AAC ACT ACG G
	hHNF4α-RT-AS	GGC AGG CTG CTG TCC TCA T
GAPDH	GAPDH-F	ACC ACA GTC CAT GCC ATC AC
	GAPDH-R	TCC ACC ACC CTG TTG CTG TA

Table S4. Primer information for pyrosequencing

Target gene	Primer type	Primers	Sequence (5′–3′)
HNF4A	F	hHNF4-PCR-F1	GTG AGT TAG GGT TTT AGT AGT TGT A
	R	hHNF4-PCR-R1	CCC AAT ACC CTC TCT ACC TT
	S	hHNF4-Seq-S1	AGG GTT TTA GTA GTT GTA AT
	S	hHNF4-Seq-S2	TTT GTA GTT TAG TTT AGT TTA TTT A
SOX17	F	hSOX17-PCR-F1	GGG GTA GGG GGA GGG GTA A
	R	hSOX17-PCR-R1	ACC CCC AAC CCA CCT AAT AAC ACT
	S	hSOX17-Seq-S1	GTG GGG TTG GAT TGG GA
	S	hSOX17-Seq-S2	GGT TTG GGA GTG GGT TTA A
	S	hSOX17-Seq-S3	GTT ATA TTT GTG TAG AAA AGG T
HNF1A	F	mehHNF1A-F1	GGG TTT TGG GGG GGT AGT
	R	mehHNF1A-R1-B	ACC CAA TAC CTA AAT CAA TAC CTC TTT ACT
	S	mehHNF1A-S1	GAG TTT GGT TTG TGT TT
	S	mehHNF1A-S2	ATT TAA GAG GTG GGG GAG G
HNF1B	F	mehHNF1B-F1	AAT GGA GTT TTT TTA GGG TAT GT
	R	mehHNF1B-R1-B	CAA ACT TCA CCT AAC CTT TAA ACT TAT T
	S	mehHNF1B-S1	TTT GAG GGT TTT TTT GGT TTA TT
	S	mehHNF1B-S2	GGA TTA AAG AGG AAT TGA GAA T
HNF6	F	mehHNF6-F1	GTA GGG GAG TAG GAG ATT TTA GAA TT
	R	mehHNF6-R1-B	ACC TTC CTT CCT CTC ACT AT
	S	mehHNF6-S1	GGG GAG AGA GGT GGT
	S	mehHNF6-S2	GGG GTA TTG AGT TTT TTT AA
CEBPA	F	mehCEBPA-F1	GAA TTA TAG GGG TAG TTT GGA GAT TAG A
	R	mehCEBPA-R1-B	TTT CAA AAC CAA AAC CAA ACC TAT C
	S	mehCEBPA-S1	GGT AGT TTG GAG ATT AGA G
	S	mehCEBPA-S2	GTT GTT TTG AGT TGT AGT TTT T
HHEX	F	mehHHEX-F1	TGA GTT AGA GGT TTT TAA ATG AAA TTA GGT
	R	mehHHEX-R1-B	ACT CAA AAC CAA ACA ATA CCC TAA ATT CC
	S	mehHHEX-S1	GAG TTA GTA GTA TTT GAA TTT TAG T
	S	mehHHEX-S2	GGG TAG TAG TTA AGG G
GATA4	F	mehGATA4-F1	TGG GTG TTT TTT GAA TTT TTA AGG A
	R	mehGATA4-R1-B	CCC CCT TCC TAT AAT CCT CAT
	S	mehGATA4-S1	ATT ATT TTT GTT TAG GAA TTA GTA
	S	mehGATA4-S2	AGA GGT TAT TTT TTT TTT TAT TGG
FOXA2 (HNF3B)	F	mehFOXA2-F1	TGG ATT TGT TGT TTT GAT AGA GAA TGA GTA
	R	mehFOXA2-R1-B	AAT CCC TAA TCC AAC CCC CTT TCC TC
	S	mehFOXA2-S1	AGA GAA TGA GTA TTG AGA G
	S	mehFOXA2-S2	ATT TGT AGG GTA TTG AGG T
GATA6	F	mehGATA6-F1	GTT TTT ATT TAG GGG ATA GGG TTT
	R	mehGATA6-R1-B	AAA ACT ACA ACC TAA ACT CCT AAT T
	S	mehGATA6-S1	ATT TTT AGA GTT TAG TTG T
	S	mehGATA6-S2	AGT TTA GAT TTA TAG TTT GGT ATT

F, forward primer; R, biotinated reverse primer; S, sequence primer.