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Supplemental Information

Wnt Inhibition Facilitates RNA-Mediated Reprogramming of Human Somatic Cells to Naive Pluripotency

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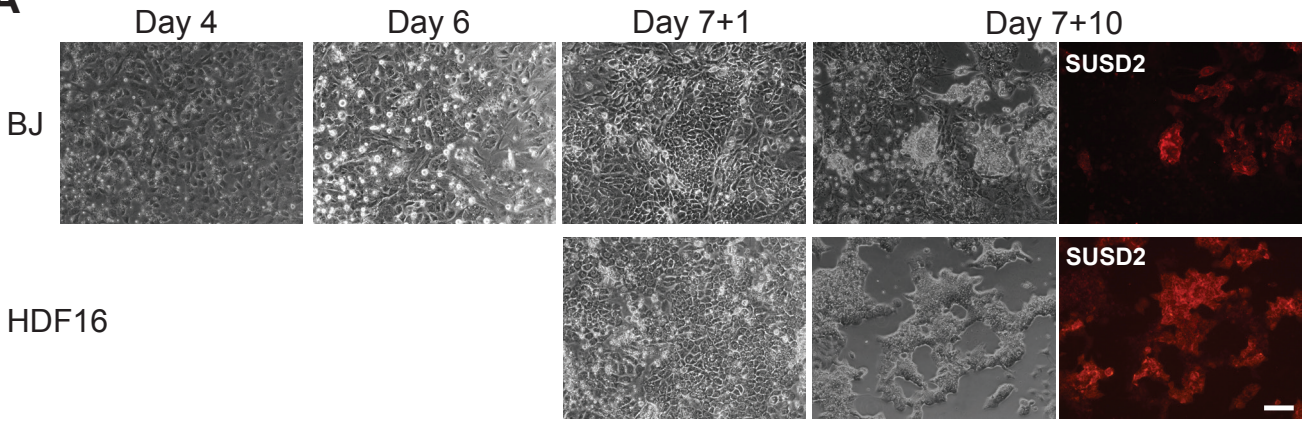
Table S2. PCR primers and related UPL probes

Supplemental protocol

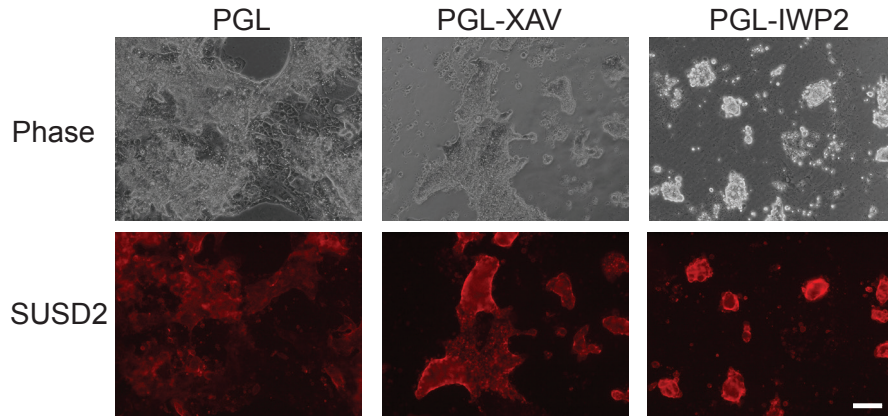
mRNA reprogramming of HDFs and EPCs.

Figure S1

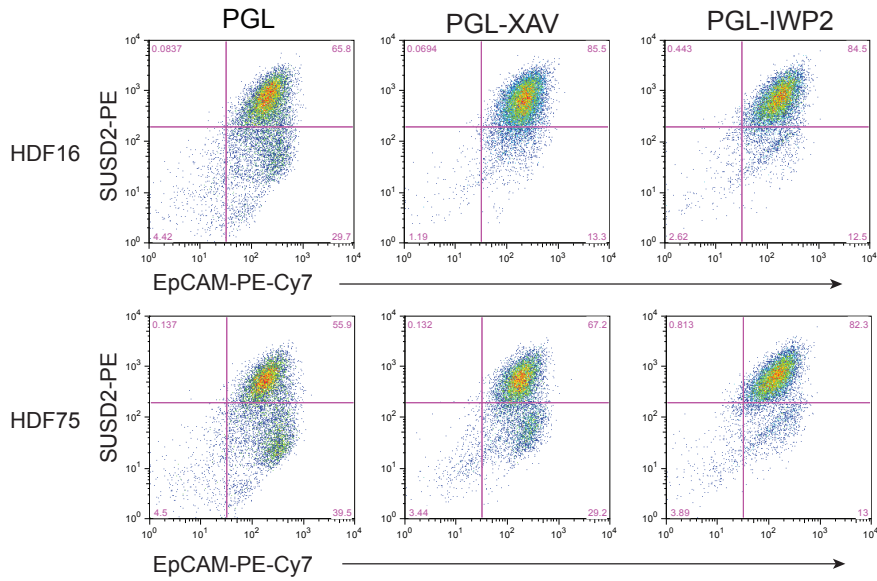
A



B



C



D

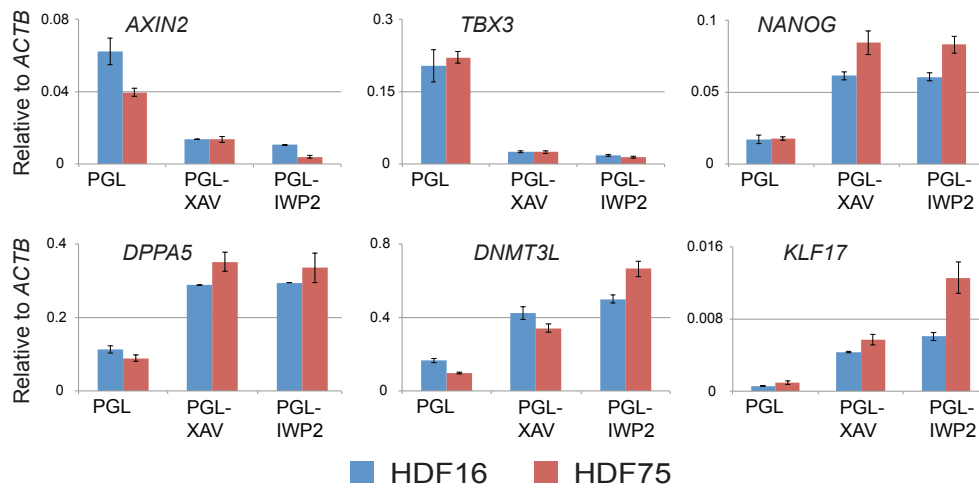


Figure S2

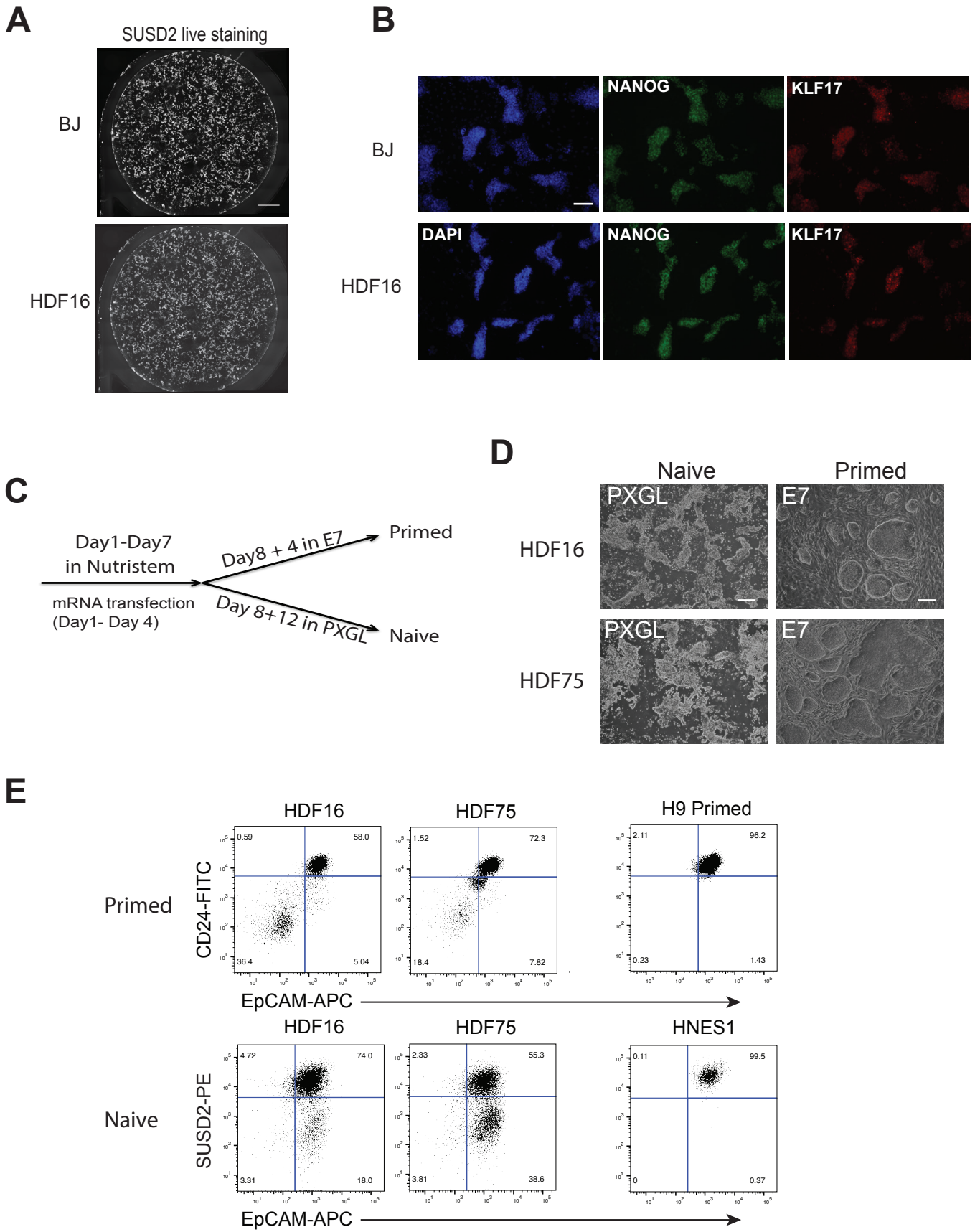
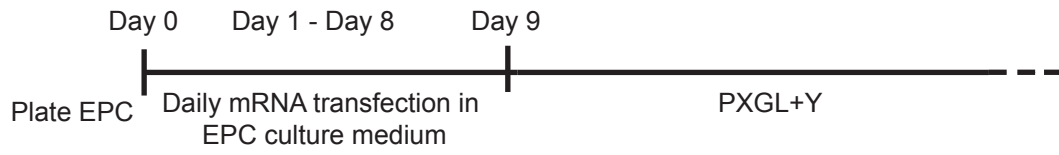
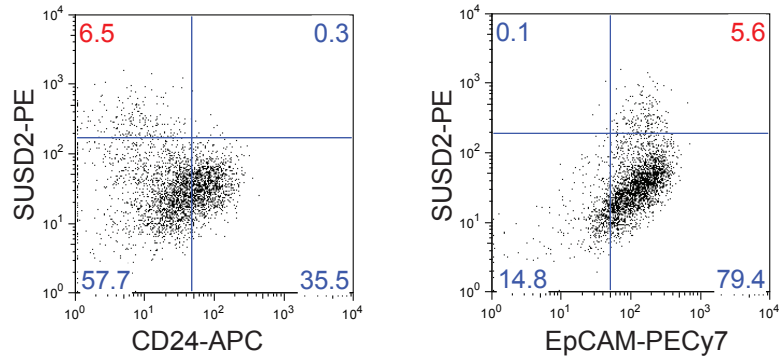


Figure S3

A

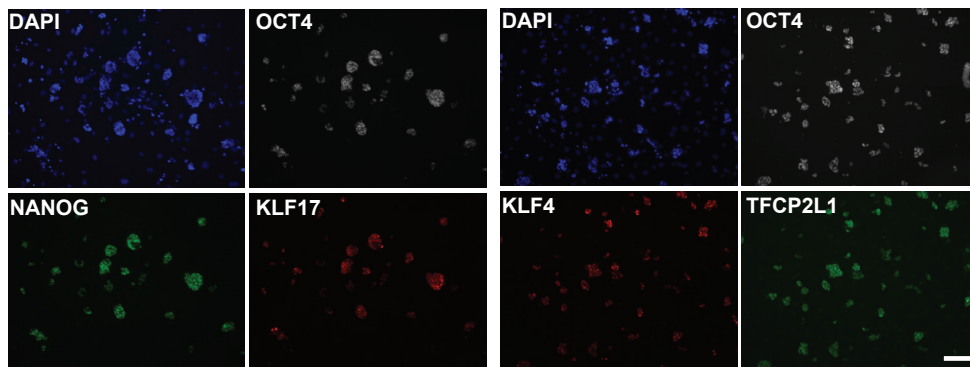


B



C

Naive iPSC reprogrammed from EPCs, P11



D

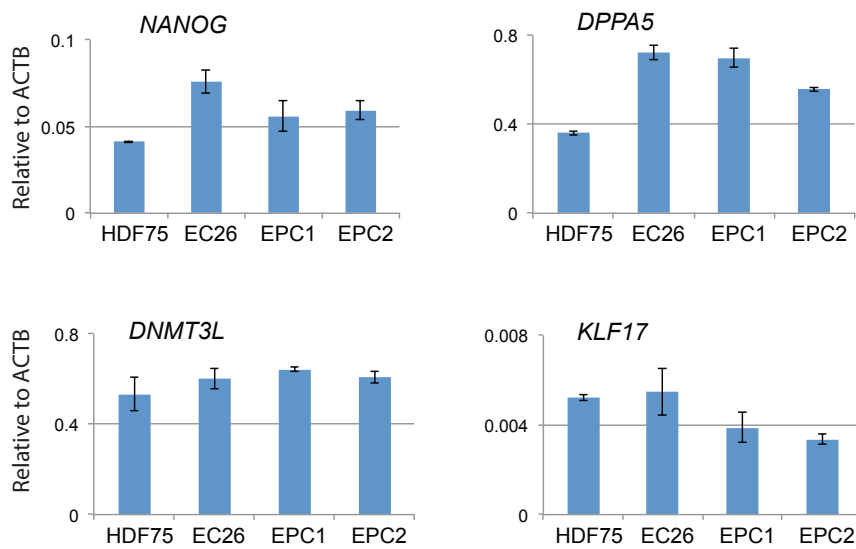


Figure S4

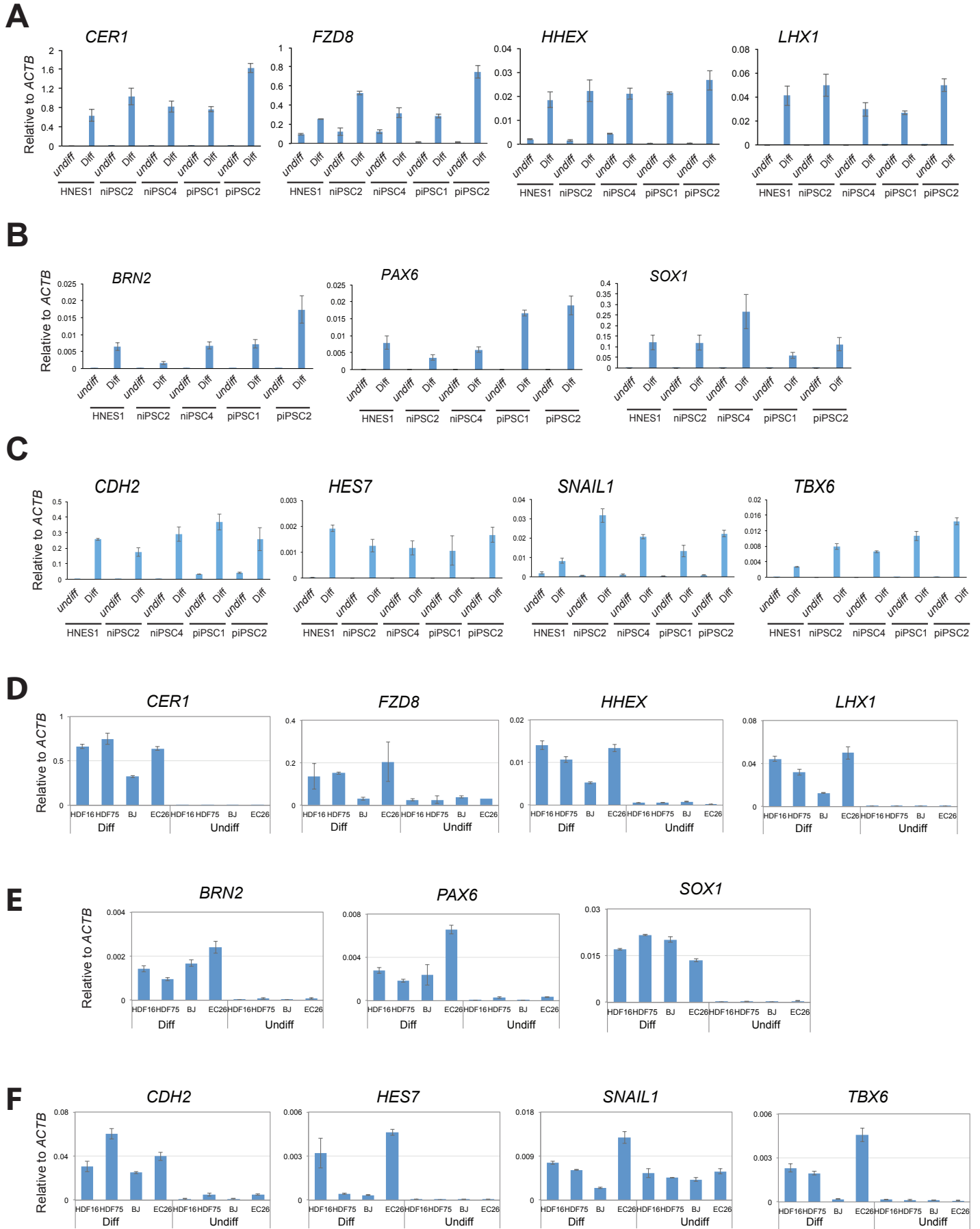
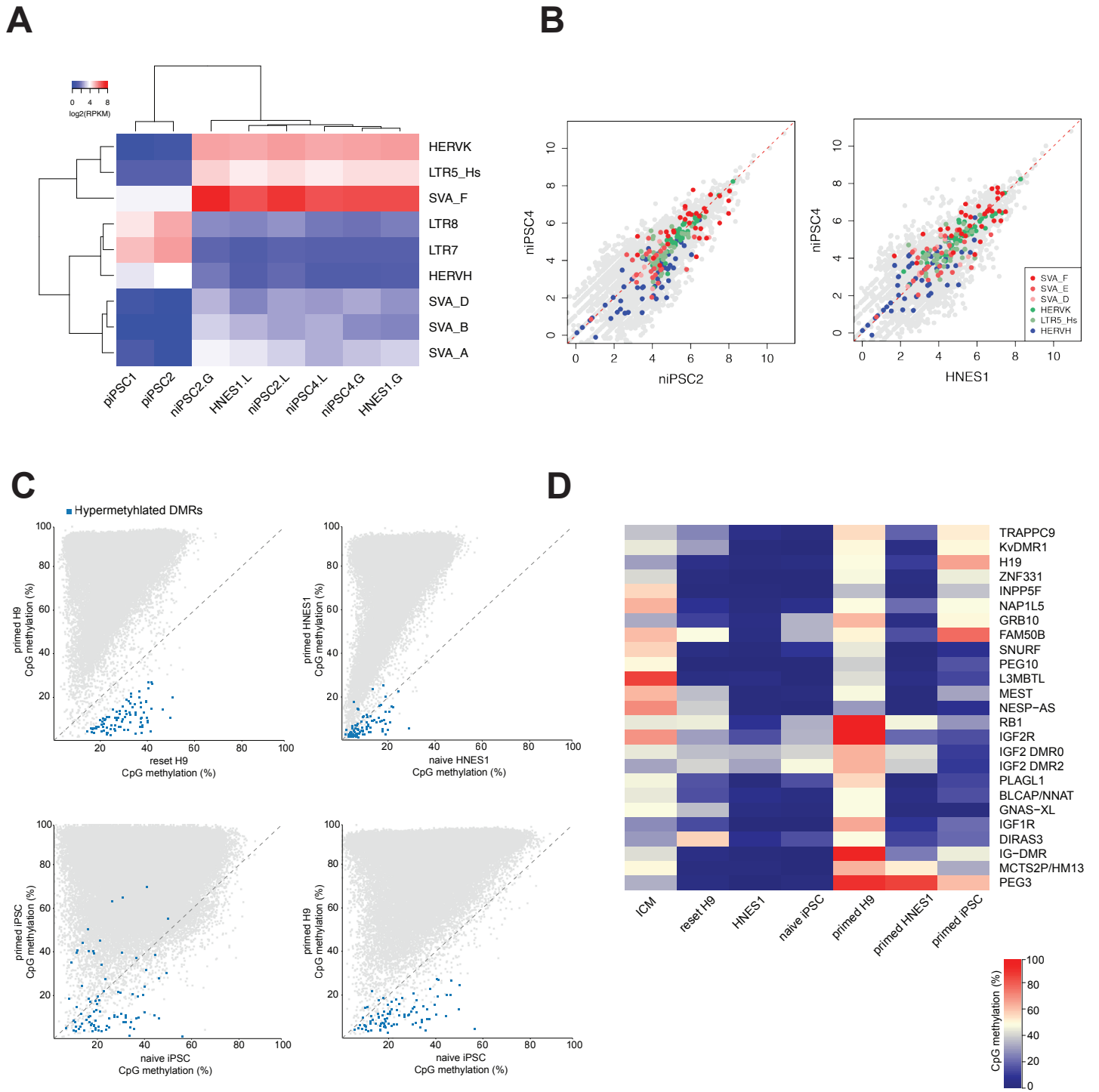


Figure S5



Supplemental Figure Legends

Figure S1. Wnt inhibition enhances naïve reprogramming by RNA, relates to Figure 1

S1A. Morphology BJ and HDF16 during reprogramming

S1B. Images of HDF75 reprogramming culture in naïve capture medium, PGL and PGL with Wnt inhibitor, XAV939 (XAV) or IWP2.

S1C. Flow cytometry analysis of EpCAM and SUSD2 expression after 12 days in PGL with XAV or IWP2.

S1D. RT-qPCR analysis of markers after 12 days in PGL based medium.

Scale bar, 100 μ M. Error bars indicate s.d. of two technical replicates.

Figure S2. Reproducibility of reprogramming, relates to Figure 2

S2A. Wells of reprogramming cultures after 13 days in PXGL, stained in situ with SUSD2-PE antibody. Scale bar, 2 mm

S2B. Immunostaining for KLF17 and NANOG after 15 days in PXGL. Scale bar, 100 μ M

S2C. Schematic of reprogramming to primed and naïve iPSCs by RNA.

S2D. Morphology of HDF16 and HDF75 after reprogramming to naïve and primed iPSCs. Scale bar, 100 μ M

S2E. Flow cytometry analysis of reprogramming to primed and naïve iPSCs. H9 primed and HNES1 are included as control for primed and naïve ESCs.

Figure S3. EPC reprogramming, relates to Figure 3

S3A. Schematic of EPC reprogramming protocol

S3B. Flow cytometry analysis of SUSD2, CD24 and EpCAM expression after three weeks in PXGL.

S3C. Immunostaining of pluripotency markers in expanded EPC-derived naïve iPSCs. Scale bar, 100 μ M

S3D. RT-qPCR analysis of three EPC derived niPSC cultures (EC26, EPC1, EPC2), comparing to HDF75 derived niPSCs. Error bars indicate s.d. of two technical replicates.

Figure S4. RT-qPCR analysis of lineage induction, relates to Figure 5

S4A. Definitive endoderm induction of naïve iPSCs (niPSC2, niPSC4) and primed iPSCs (piPSC1, piPSC2)

S4B. Neuroectoderm induction of naïve iPSCs (niPSC2, niPSC4) and primed iPSCs

S4C. Paraxial mesoderm induction of naïve iPSCs (niPSC2, niPSC4) and primed iPSCs

S4D. Definitive endoderm induction of naïve iPSCs derived from HDFs and EPC

S4E. Neuroectoderm induction of naïve iPSCs derived from HDFs and EPC

S4F. Paraxial mesoderm induction of naïve iPSCs derived from HDFs and EPC

Error bars indicate s.d. of three technical replicates.

Figure S5. Analysis of transposon element expression and CpG methylation, relates to Figure 6

S5A. A heatmap showing the expression of known naïve and primed-specific TEs (average expression of all TE loci of the subfamily).

S5B. Scatter plots showing the expression of TE elements in niPSC2, C4 and HNES1 cells. TE elements from representative TE subfamilies that are differentially expressed between naïve and primed cells (Theunissen et al., 2016, Guo et al., 2017) are highlighted.

S5C. Scatter plots of CpG methylation percentages over tiles spanning 20 kb. Regions with >10% gain in CpG methylation in reset H9-NK2 cells⁹ compared to conventional primed H9 cells are highlighted in blue in all scatterplots.

S5D. Averaged CpG methylation of known DMRs of imprinted maternal and paternal genes.

Table S1. Taqman assays, relates to experimental procedures

Gene	TaqMan Assay ID
<i>ACTB</i>	Hs01060665_g1
<i>NANOG</i>	Hs02387400_g1
<i>OCT4</i>	Hs01654807_s1
<i>KLF4</i>	Hs00358836_m1
<i>KLF17</i>	Hs00703004_s1
<i>TFCP2L1</i>	Hs00232708_m1
<i>DPPA3</i>	Hs01931905_g1
<i>DPPA5</i>	Hs00988349_g1
<i>DNMT1</i>	Hs00945875_m1
<i>DNMT3A</i>	Hs01027166_m1
<i>DNMT3B</i>	Hs00171876_m1
<i>DNMT3L</i>	Hs01081364_m1
<i>SOX2</i>	Hs01053049_s1
<i>PRDM14</i>	Hs01119056_m1

Table S2. PCR primers and related UPL probes, relates to experimental procedures

Gene	Forward Primer	Reverse Primer	UPL probe
<i>SOX1</i>	accaggccatggatgaag	cttaattgctgggaattgg	37
<i>PAX6</i>	ggcacacacacattaacacactt	ggtgtgtgagagcaatttcag	9
<i>BRN2</i>	aataaggcaaaaggaaagcaact	caaacacatcattacacctgct	57
<i>HHEX</i>	cggacggtgaacgactaca	agaaggggctccagagtagag	61
<i>LHX1</i>	atgcaacctgaccgagaagt	caggtcgctaggggagatg	80
<i>CER1</i>	gccatgaagtacattgggaga	cacagcctctgtgggtatag	41
<i>FZD8</i>	cgccacgcgtaatttct	ccggttctggaaccacac	19
<i>CDH2</i>	tgacagatgtggacaggat	ccacaaacatcagcacaagg	15
<i>SNAI1</i>	gcgagctgcaggactctaat	cggtggggtgaggatct	62
<i>HES7</i>	gcagcctggaagagctga	acggcgaactccaatatctc	78
<i>TBX6</i>	gaacggcagaaactgtaagagg	gtgtgtctccgctccatag	5
<i>TCF15</i>	tgtccgggacactctgg	caggctgaatggatcctcac	80
<i>ZEB1</i>	agcacttaagaattcacagtggag	catttctactgcttatgtgtgagc	36
<i>OTX2</i>	gggtatggacttctgcac	ccgagtgaacgtcgtcct	81
<i>TBX3</i>	ggtcattaccaagtcgggaag	tcagcagctataatgtccatcaa	26

Supplemental protocol

1. Reprogramming human dermal fibroblasts to naïve pluripotent stem cells

Materials

HDFa (human dermal fibroblast, adult)
Irradiated mouse embryonic fibroblast (MEF)
StemRNA 3rd Gen Reprogramming Kit (Stemgent,00-0076)
Lipofectamine® RNAiMAX™ (Thermo Fisher Scientific, 13778150)
Geltrex (hESC-Qualified, Thermo Fisher Scientific, A1413302)
SUSD2 (PE conjugate) (BioLegend, 327406)

Culture media

Fibroblast culture medium
DMEM high glucose (Merck, D5546), FBS (10%, Merck, F0804), L-glutamine (2 mM, Thermo Fisher Scientific, 25030-024), 2-mercaptoethanol (100 µM, Merck, M3148)

Modified E7 medium
Home made E6 basal medium (Chen et al., 2011) supplemented with 10 ng/mL FGF2 (prepared in-house).

NutriStem™ hPSC XF Medium (Biological Industries, 01-0005)

Naïve hPSC medium, PXGL
N2B27 medium supplemented with MEK inhibitor PD0325901 (1 µM), tankyrase inhibitor XAV939 (2 µM), aPKC inhibitor Gø6983 (2 µM), human LIF (10 ng/mL, prepared in-house), and optionally Rho-kinase inhibitor Y-27632 (10 µM).

Note. The quality of N2B27 medium is of paramount importance and batches should be tested on naïve hPSCs if available, or else on mouse ES cells as described (Mulas et al., 2019)

Protocol

1: Day 0: Dissociate HDFs with TrypLE. Collect dissociated cells, pellet at 300g for 3 minutes and resuspend in fibroblast culture medium. Count cells and plate at a density of $1 \times 10^4/\text{cm}^2$ on tissue culture plates pre-coated with Geltrex (1 µL/cm²).

2: Day 1: Switch to modified E7 medium and perform mRNA transfection following recommendation of StemRNA™-NM Reprogramming Kit protocol.

3: Day 2-4: Repeat mRNA transfection daily. **Note:** If excessive cell death is observed after mRNA transfection, it is recommended to plate fibroblasts at higher density. Alternatively, reducing mRNA transfection to 3 days is usually sufficient to generate at least 20 naïve colonies/4-well dish.

4: Day 5-6: Refresh culture with modified E7 medium. NutriStem™ can be used as an alternative medium. **Note,** by day 6, patches of cells with epithelial morphology should become apparent, indicating reprogramming has been initiated.

5: Day 7: Switch to human naïve culture medium, PXGL, and maintain for about two weeks. SUSD2 positive colonies should appear after 7-10 days in PXGL medium and can be visualised by live cell staining (Brendenkamp et al., 2019). Rock inhibitor (Y-27632) may be added to PXGL medium during reprogramming. **Note,** transfer to PXGL medium can be varied between Day 6 to Day 9. Delaying medium switch beyond Day 10 will significantly reduce reprogramming efficiency.

2. Reprogramming human blood outgrowth derived endothelial progenitor cells (EPC) to naïve pluripotent stem cells

Materials

EPC reprogramming uses the same materials as HDFA reprogramming, if not specified otherwise.

EPC medium (50 mL)

Endothelial Cell basal medium (PromoCell, c-22210) or EBM-2

Endothelial Cell basal medium (Lonza, cc-3156) 40-45 mL

5-10 mL heat-inactivated FBS (heat-inactivation is not necessary) (20% FBS when thawing the EPCs, 10% FBS for regular culture)

Hydrocortisone (Lonza, cc-4112A) 16 µL

hFGF-B (Lonza, cc-4113A) 160 µL

VEGF (Lonza, cc-4114A) 16 µL

R3-IGF-1 (Lonza, cc-4115A) 16 µL

hEGF (Lonza, cc-4317A) 16 µL

Ascorbic acid (Lonza, cc-4116A) 16 µL

GA-1000 (Lonza, cc-4381A) 16 µL

Reprogramming EPCs

1. EPCs are cultured according to (Ormiston et al., 2015). Cells are grown in 50 µg/mL Collagen I coated T-75 flasks with endothelial growth medium supplemented with growth factors with 10-20% FBS and without heparin (EPC medium).
2. When EPCs reach 80-90% confluence, cells are dissociated with TrypLE then resuspended at 2×10^6 /mL in EPC medium.
3. Add $1-2 \times 10^5$ EPCs (0.5 mL) per well of a 4-well dish (coated with 2.4 µg/mL Laminin 511 (iMatrix-511, Reprocell, T303) at least 1 h before plating).
4. Next day, refresh with EPC medium about one hour before transfection. We normally do mRNA transfection after 5pm.
5. Perform mRNA cocktail transfection as detailed in *StemRNA 3rd Gen Reprogramming Kit*.
6. Next morning usually before 9-9.30am, refresh culture with fresh EPC medium; then late in the afternoon repeat mRNA transfection.
7. Repeat daily until Day 9.
8. Day 9, exchange to human naïve medium with 10 µM Y-27632 for naïve iPSC induction.
9. Following 15-20 days culture in naïve medium, naïve colonies should be readily identifiable by refractile dome-shaped morphology. At this point, naïve colonies can be picked or bulk passaged.

3. Passaging naïve cells

1. Dissociate culture with Accutase or TrypLE Express (about 5-10 minutes at 37°C).
2. Pellet cells at 300g for 3 min. Aspirate and re-suspend cells in PXGL with Y-27632 (PXGLY).
3. Aliquot cells to plates coated with MEF feeders in PXGL plus Y-27632. We recommend adding Geltrex (0.5 µL per cm²) to cells at the time of passaging.
4. The next day, top up wells with fresh PXGL medium (without Y-27632). Subsequently, change half-medium daily until passaging. This normally takes 4-5 days culture in PXGL medium at a split ratio of 1:4 to 1:8. Do not let colonies grow too large.

Note: A stable naïve iPSC culture will present with more than 80% SUSD2+CD24- cells after three passages in PXGL medium. If not, picking colonies or sorting for SUSD2+CD24- may be necessary to establish a stable iPSC culture.

References:

Bredenkamp, N., Stirparo, G.G., Nichols, J., Smith, A., and Guo, G. (2019). The Cell-Surface Marker Sushi Containing Domain 2 Facilitates Establishment of Human Naïve Pluripotent Stem Cells. *Stem Cell Reports* 12, 1212-1222.

Chen, G., Gulbranson, D.R., Hou, Z., Bolin, J.M., Ruotti, V., Probasco, M.D., Smuga-Otto, K., Howden, S.E., Diol, N.R., Propson, N.E., *et al.* (2011). Chemically defined conditions for human iPSC derivation and culture. *Nat Methods* 8, 424-429.

Mulas, C., Kalkan, T., von Meyenn, F., Leitch, H.G., Nichols, J., and Smith, A. (2019). Defined conditions for propagation and manipulation of mouse embryonic stem cells. *Development* *146*, dev173146

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