Stem Cell Reports, Volume *8*

Supplemental Information

Differentiation-Defective Human Induced Pluripotent Stem Cells Reveal Strengths and Limitations of the Teratoma Assay and In Vitro Pluripotency Assays

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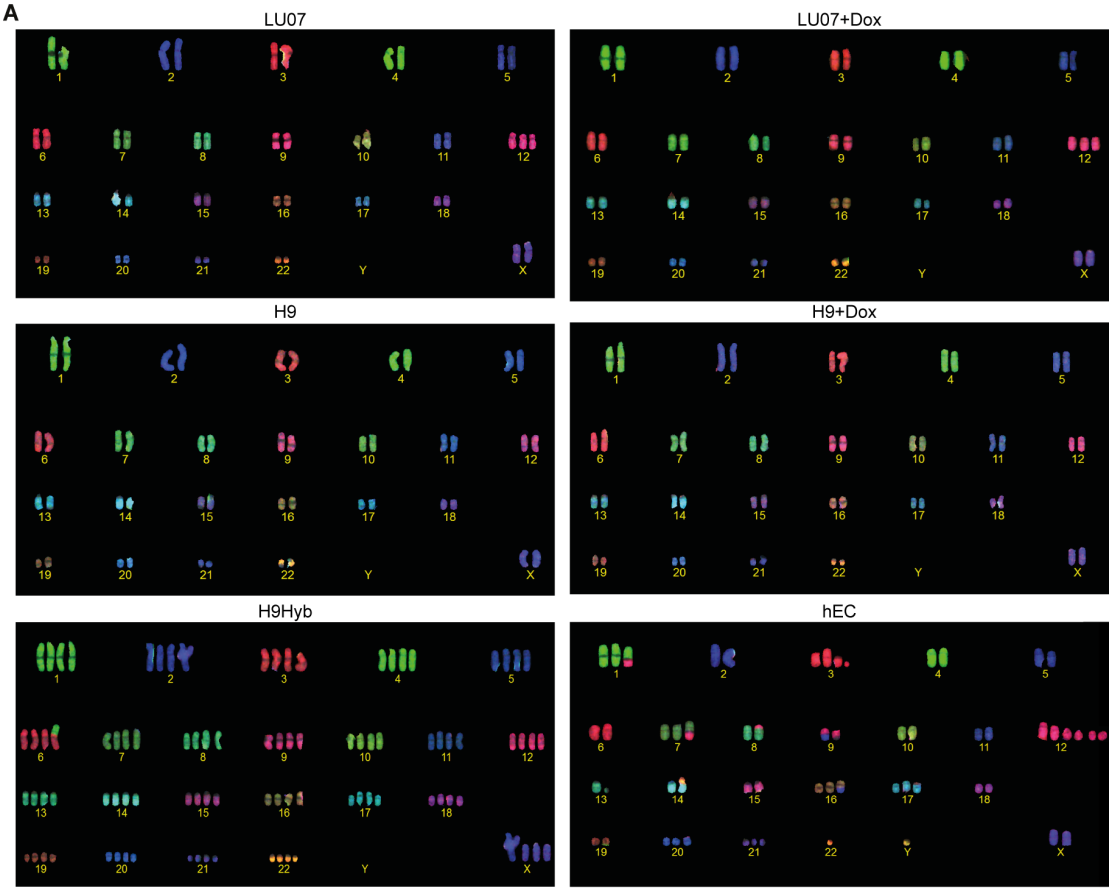
A



B

Cell line	Passage number	Karyotype
LU07	37	46,XX[14], 47,XX+12[1]
LU07+Dox	37	46,XX[16], 47,XX+12[4]
H9	40	46,XX[20]
H9+Dox	40	46,XX[20]
H9Hyb	18	92,XXXX,der(6)t(1;6)[20]
hEC	39	57~61,XXY,+der(1)t(1;9),del(3),+der(7)t(7;9),der(8)t(8;12),der(9)t(5;9), +i(12)(p)x4,del(13),der(14)t(2;14),rob(14;22),+der(16)t(2;16),+der(17)t(17;19), +20[cp20]

Figure S1. *Karyotyping of undifferentiated cells used for in vivo injections and PluriTest*, related to Fig. 1. **(A)** Representative COBRA karyograms. **(B)** Summary of karyotypes found by COBRA analysis. Number of metaphases indicated in brackets.

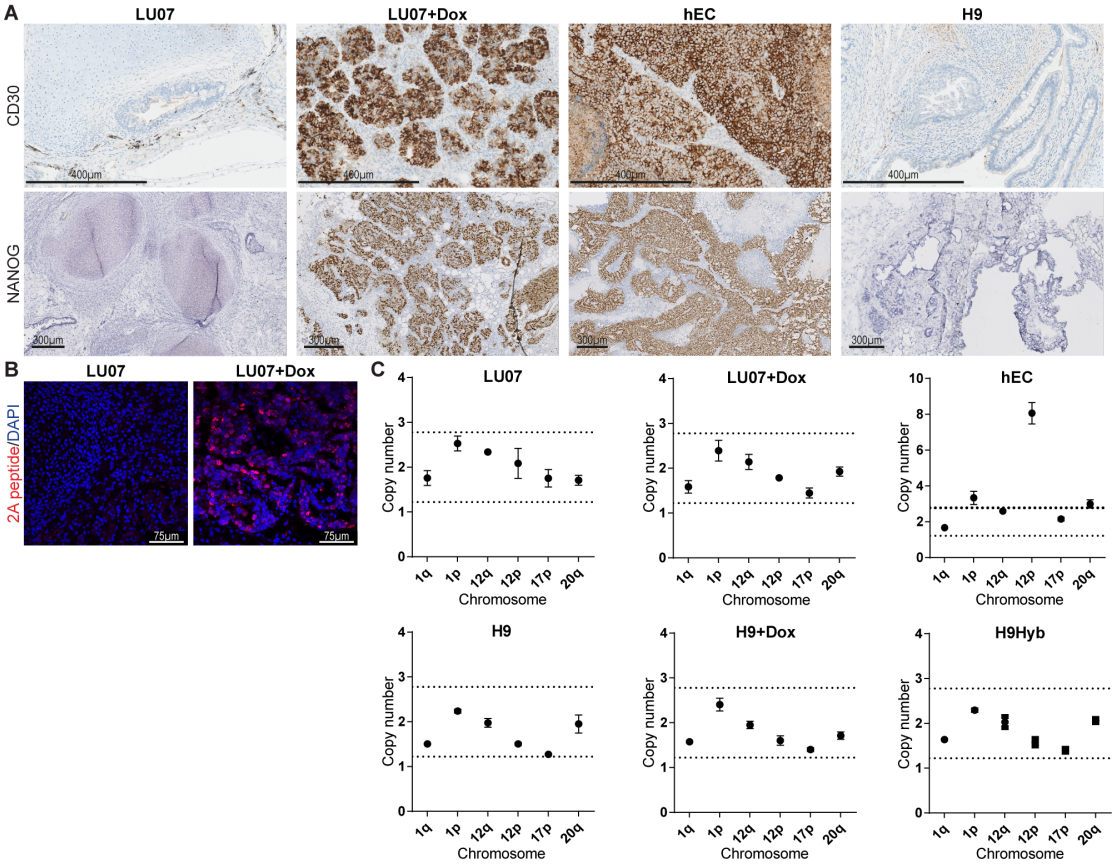


Figure S2. *Immunohistochemistry staining and karyotype analysis of xenografts*, related to Fig. 2. **(A)** Representative images of CD30 and NANOG expression in xenograft sections. Scale bars: 400µm and 300µm, respectively. **(B)** IF staining of 2A peptide in xenograft sections: nuclei were stained with DAPI. Scale bars: 75µm. **(C)** Average copy numbers (± SEM) for selected chromosomal regions in xenografts as detected by qPCR. Xenografts analyzed: LU07/H9/hEC: n=2, LU07+Dox/H9+Dox/H9Hyb: n=3. Dotted lines represent cutoff levels calculated as three SDs of the copy number values of the calibrator samples. Copy number values within this range are considered normal. Note that H9Hyb appears as diploid, since tetraploidy concerns all genes including the housekeeping gene.

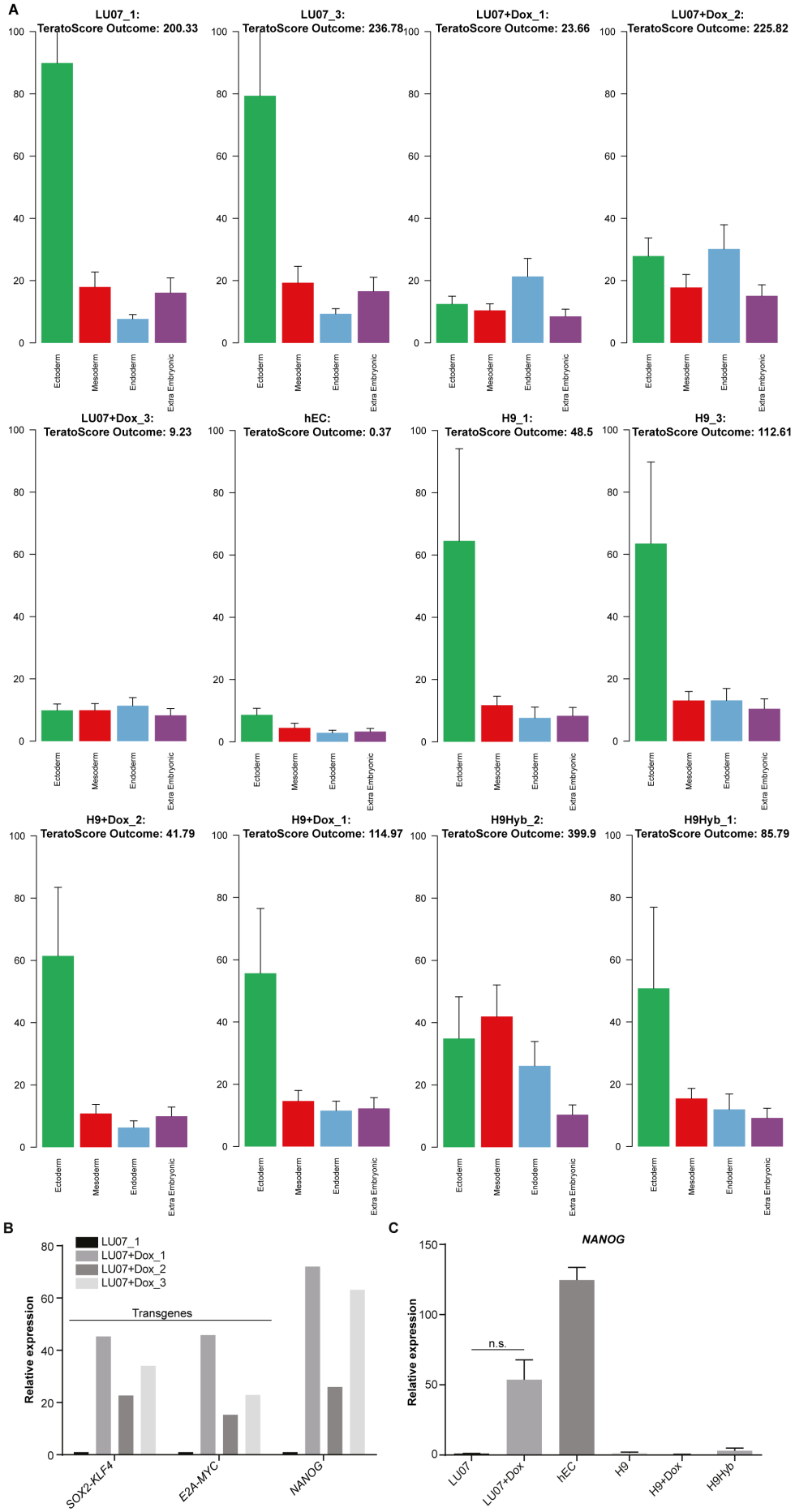

A
LU07_1:
TeratoScore Outcome: 200.33
LU07_3:
TeratoScore Outcome: 236.78
LU07+Dox_1:
TeratoScore Outcome: 23.66
LU07+Dox_2:
TeratoScore Outcome: 225.82
LU07+Dox_3:
TeratoScore Outcome: 9.23
hEC:
TeratoScore Outcome: 0.37
H9_1:
TeratoScore Outcome: 48.5
H9_3:
TeratoScore Outcome: 112.61
H9+Dox_2:
TeratoScore Outcome: 41.79
H9+Dox_1:
TeratoScore Outcome: 114.97
H9Hyb_2:
TeratoScore Outcome: 399.9
H9Hyb_1:
TeratoScore Outcome: 85.79
Ectoderm
Mesoderm
Endoderm
Extra Embryonic
B
LU07_1
LU07+Dox_1
LU07+Dox_2
LU07+Dox_3
Transgenes
Relative expression
SOX2-KLF4
E2A-MYC
NANOG
C
NANOG
n.s.
Relative expression
LU07
LU07+Dox
hEC
H9
H9+Dox
H9Hyb

Figure S3. *Detailed TeratoScores and pluripotency marker analysis in xenografts*, related to Fig. 3. **(A)** Individual TeratoScores for ectoderm (green), mesoderm (red), endoderm (blue) and extraembryonic tissue (purple). Samples are identical to samples of Fig. 3. **(B)** Expression levels of transgenic *SOX2-KLF4* and *2A peptide-c-MYC* and endogenous *NANOG* in TeratoScore xenografts as determined by qPCR (± SEM) **(C)** Average endogenous *NANOG* expression levels (± SEM) in xenografts as determined by qPCR (n=3).

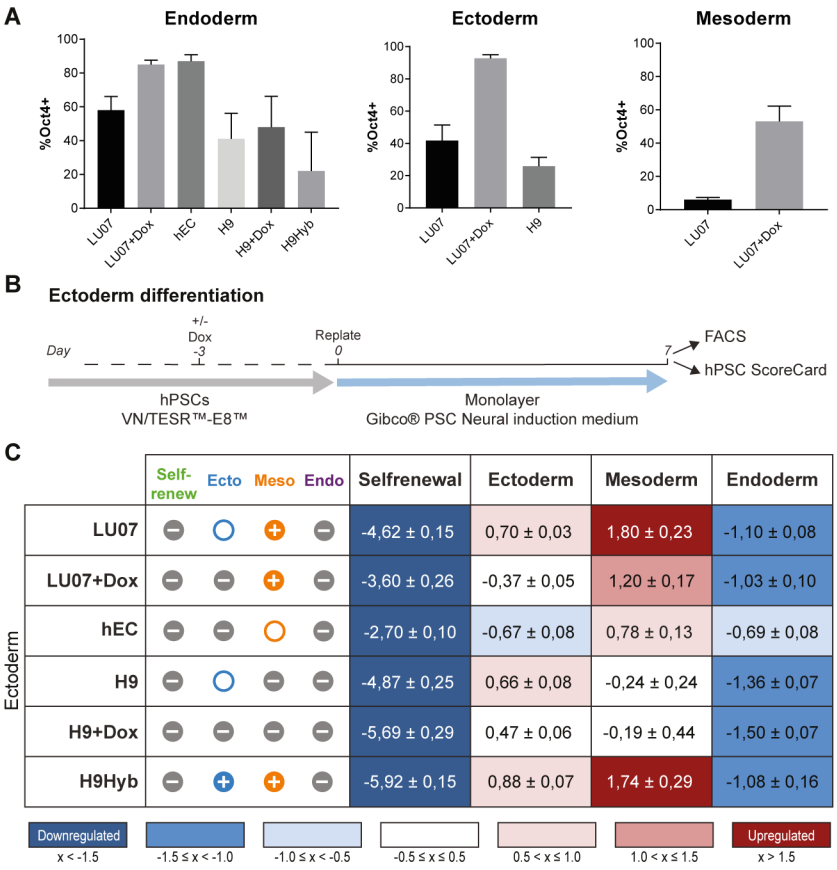


Figure S4. *FACS data for in vitro differentiations. Schematics and results of the monolayer ectodermal differentiation protocol*, related to Fig. 4. **(A)** Percentage of Oct4^{+} cells measured by FACS at the end of endodermal (left panel), ectodermal (middle panel, StemDiff Neural Induction system) and mesodermal differentiation. Data represented as average ±SEM. Samples are identical to samples of Fig. 4. **(B)** Schematics of the monolayer differentiation procedure towards ectoderm. **(C)** Average hPSC Scorecard results for the ectodermal monolayer differentiations displayed with icons: "**+**" (positive), "O" (borderline) or "**–**" (negative) and colour code: green (self-renewal), blue (ectoderm), orange (mesoderm), purple (endoderm). Icons represent the average of biological repeats (LU07/LU07+Dox: n=5, H9/H9+Dox: n=4, hEC: n=3, H9Hyb: n=2, independent experiments). Right: Average scores (±SEM) of the same differentiations. Blue: downregulated, white: unchanged, red: upregulated.

Table S1. *PluriTest Scores*, related to Fig. 5.

Sample	Pluripotency Score	Novelty Score	Passage number
LU07_1	27.56	1.32	46
LU07_2	25.07	1.31	47
LU07_3	27.88	1.33	37
LU07_4	27.20	1.25	42
LU07+Dox_1	25.71	1.36	47
LU07+Dox_2	28.75	1.31	37
LU07+Dox_3	30.80	1.48	46
LU07+Dox_4	30.28	1.29	42
hEC_1	21.19	1.91	43
hEC_2	13.47	1.97	45
hEC_3	15.58	1.91	40
H9_1	23.59	1.27	46
H9_2	24.24	1.28	47
H9_3	27.98	1.39	40
H9+Dox_1	25.39	1.34	46
H9+Dox_2	24.75	1.30	47
H9+Dox_3	25.66	1.37	40
H9Hyb_1	25.77	1.34	20
H9Hyb_2	22.8	1.38	18
H9Hyb_3	22.49	1.46	21

Table S1. Pluripotency and Novelty Scores (PluriTest) for undifferentiated cells at the indicated passage numbers. Samples are identical to samples of Fig. 5.

Supplemental Experimental procedures

Cell lines

The H9 human embryonic stem cell (hESC) line was purchased from WiCell. The H9 ESC hybrid line (clone: Hybrid_6) was a generous gift from Prof. M. Zenke, RWTH, Aachen, Germany. Human embryonal carcinoma (hEC) cells 2102Ep were kindly provided by Prof. P. Andrews, Sheffield, UK. The hiPSC line LUMC007iCTRL01 was generated from skin fibroblasts using a doxycycline (Dox)-inducible lentivirus encoding mouse cDNAs for *OCT3/4*, *SOX2*, *KLF4* and *c-MYC* separated by three different 2A peptides (TetO-FUW-OSKM) and a lentivirus carrying the tetracycline controllable transactivator (FUW-M2rtTA, plasmids from Addgene) (Fig. 1A). Briefly, three days post transduction of 125000 fibroblasts with the lentiviruses, transduced cells were plated on mouse embryonic fibroblasts (MEFs) and maintained in hESC media supplemented with 1 μg/ml Dox (Sigma) until the appearance of hiPSC colonies. After picking, hiPSC colonies were expanded without Dox on Matrigel (Corning) in mTESR1 media (Stem Cell Technologies).

Cell culture

All hPSCs were maintained on Vitronectin-XF in TESR-E8 media (Stem Cell Technologies) with daily media changes and passaged once a week as small aggregates according to the manufacturer's protocol. LUMC007iCTRL01 was cultured in the absence of Dox unless otherwise stated. hPSCs which had been maintained in other cell culture systems previously were adapted to Vitronectin-XF and TESR-E8 for at least three passages before experiments were performed. hECs were maintained in DMEM/F12 media containing 10% FCS and passaged twice a week with 0.05% Trypsin. Cell culture reagents were from Life Technologies.

***In vitro* differentiation assays**

Monolayer differentiation of hPSCs into neural stem cells with Neural induction medium (Life technologies) was performed according to the manufacturer's protocol. Briefly, one day after passaging cells as small aggregates, Neural Induction media was added and changed every other day until day 7. When indicated, 2 ug/ml Dox was added two days prior to splitting, during the plating step and during differentiation at each media change, respectively.

Neural progenitor cells (NPCs) were generated by using the EB protocol from Stem Cell Technologies (Stemdiff Neural System). At the day of passaging, 2.25 - 3x10^6 single PSCs in Neural induction media (NIM) containing Rock inhibitor (LC laboratories) were added to one well of an Aggrewell 800 plate for formation of EBs. Partial media changes were performed as described in the manufacturer's instructions until day 5, when EBs were replated onto Matrigel-coated cultureware and maintained in NIM until day 9. When indicated, Dox was added two days prior to preparation of single cells, during EB formation and throughout the differentiation at each media change, respectively.

For differentiation into endoderm with the Stemdiff Definitive Endoderm Kit (TESR-E8 optimized, Stem Cell Technologies), undifferentiated hPSCs were cultured for two days in pre-differentiation media and single cells were plated onto Vitronectin-XF so that they reached confluency

the next day. Twenty-four hours post-plating, endodermal differentiation was initiated and performed during 5 days according to the manufacturer's protocol. When indicated, Dox was added one day after plating single cells and at least 6 hours before starting the differentiation into endoderm. During differentiation, Dox was added at each media change.

Mesoderm differentiation was performed using STEMdiff mesoderm induction medium (Stem Cell Technologies) according to manufacturer's protocol. Briefly, undifferentiated hPSCs were replated as single cells 32h prior to the start of differentiation. Mesodermal induction medium was applied to the cells for 5 days. When indicated, Dox was added 24h prior to the start of differentiation. At the end of each differentiation, cells were analyzed by FACS or processed for RNA isolation.

For *in vitro* differentiations undifferentiated cells with the following range of passage numbers were used: H9(+Dox) (37-56), LU07(+Dox) (38-54), H9Hyb (18-37), hEC (56-95).

Karyotyping with the COBRA assay and by qPCR

Karyotyping analysis of undifferentiated cells was performed as previously described (Szuhai and Tanke, 2006). Passage numbers are indicated in Fig. S1B. For analysis of aneuploidies in teratomas DNA was isolated using the NucleoSpin kit (Macherey-Nagel) including a digestion step with RNase (Invitrogen) and the qPCR assay was performed as described (Baker et al., 2016).

FACS analysis

Single undifferentiated or differentiated cells were prepared with Gentle Cell Dissociation Reagent (Stem Cell Technologies) or 0.05% trypsin (Life Technologies), respectively. Cells were fixed and permeabilized using the FIX & PERM® Cell Fixation & Permeabilization Kit (Invitrogen) according to manufacturer's protocol. During permeabilization, cells were stained with the appropriate antibody and subsequently analyzed using the MACSQuant® VYB (Miltenyi Biotech). Antibodies were: OCT4-Alexa488 (Millipore) or OCT3/4-Isoform A-PE (Miltenyi Biotec), both 1:50 or isotype controls; mouse IgG1-Alexa Fluor® 488 (eBioscience) or mouse IgG1-PE (Miltenyi Biotec).

Teratoma assay

8-10 weeks old male NSG mice (NOD.Cg-Prkdc^{scid} Il2rg^{tm1Wjl}/SzJ, Charles River) were used for injections. Animal experiments were approved by the Leiden University Medical Center (LUMC) animal ethics committee. The LUMC is an institutional license holder according to the Dutch Law on animal experimentation.

When indicated the drinking water contained Dox (2mg/ml, Sigma-Aldrich) and Sucrose (10 mg/ml, Sigma-Aldrich) one week prior to and after injections, which was changed every second day until mice were sacrificed. Drinking water without Dox contained Sucrose only. The passage numbers of the cells used for injections are indicated in Fig. S1B. Single hPSCs were prepared with Gentle Cell Dissociation Reagent (Stem Cell Technologies) according to the manufacturer's protocol. hEC cells were prepared using trypsin 0.05% (Life Technologies, 5 min, 37°C). When indicated, cells were cultured in the presence of Dox (2 ug/ml) three days prior to injection. 1x 10^6 cells were resuspended in 200 µl of cold TESR-E8 (+Dox when indicated) or hEC media mixed with Matrigel (1:1), respectively

and injected subcutaneously in the flank region. Tumor growth was monitored weekly by palpation and mice were sacrificed when teratomas reached a volume ≤ $2cm^3$. The resulting teratomas were stored in RNAlater (Ambion) or fixated for cryosectioning: 4h in 4% PFA (RT), ≥2h 15% Sucrose-PBS (RT), O/N 30% Sucrose-PBS solution (4°C), Tissue-Tek® OCT compound (Sakura® Finetek) (-80°C).

Immunohistochemistry and IF staining

8 µm frozen sections were processed for hematoxylin and eosin (HE) staining according to standard procedures. For IF staining, sections were postfixated with 2% PFA for 10 minutes, permeabilized for 8 minutes with PBS/0.1% Triton-X-100 (Sigma-Aldrich) and incubated with blocking solution (4% normal swine serum/PBS) for 1h (RT). Primary antibodies were applied O/N (4°C), followed by the secondary antibodies for 1 hour (RT). DNA counterstaining was performed with DAPI (1:1000, Invitrogen). Images were taken using a Leica TCS SP5 confocal microscope.

For IF staining of cultured cells, cells were fixed with 2% PFA/PBS for 30 min at RT. Consecutive procedures were as described above.

For immunohistochemistry stainings slides were fixated in cold acetone for 5 minutes. CD30 and Nanog antibodies were incubated O/N (RT); counterstaining was performed with hematoxylin. The CD30 staining was performed automatically using a Ventana BenchMark ULTRA machine (Ventana Medical System Inc.) using the Amplification kit (Ventana Medical System, 760-080) and the UltraView Universal DAB Detection kit (Ventana Medical System, 760-500). The Nanog staining was performed using the Vectastain Elite ABC HRP kit (peroxidase, standard, PK 6100, Vector Laboratories Inc.) and DAB (3 3'-diaminobenzidine tetrahydrochloride, Sigma-Aldrich 32750-25 G-F). Slides were scanned using a Pannoramic Digital slide Scanner (3DHISTECH Ltd.). Antibodies are listed in the table below.

Table of antibodies used for IF and immunohistochemistry stainings

1st Antibody	**Dilution**	**2nd Antibody**	**Dilution**
Alpha-1 Feto-protein (AFP) rabbit IgG (2011200530, Quartett)	1:25	donkey anti-rabbit IgG-Alexa 488 (A-11031, Invitrogen) or for H9Hyb samples: donkey anti-rabbit IgG - Alexa 647 (A-31573, Invitrogen)	1:500 1:250
β-III-tubulin mouse IgG2a (MMS-435P, Covance)	1:4000	goat anti-mouse IgG - Alexa 568 (A-11031, Invitrogen)	1:500
CD-30 (for IHC) mouse IgG (790-4858, Roche)	Not indicated Antibody specifically produced for a VENTANA BenchMark IHC automated slide stainer	Amplification kit: (Ventana Medical System, Rocklin, CA, 760-080) Detection system: ultraView Universal DAB Detection kit (Ventana Medical System, Rocklin, CA, 760-500)	Not indicated Antibody specifically produced for a VENTANA BenchMark IHC automated slide stainer

CD-31 (PECAM) mouse IgG1(AB525, DAKO)	1:20	goat anti-mouse IgG1 - Alexa 568 (A-21124, Invitrogen)	1:250
NANOG (for IF) mouse IgG1 (sc-293121, Santa Cruz)	1:150	donkey anti-mouse IgG-Alexa 488 (A-21202, Invitrogen) or goat anti-mouse IgG-Alexa 568 (A-21124, Invitrogen)	1:250
NANOG (for IHC) goat IgG (AF1997, R&D Systems)	1:200	rabbit anti-goat polyclonal (E0466 Dako) ABC complex: Vectastain Elite ABC HRP kit (peroxidase, standard), PK 6100, Vector Laboratories Inc. Substrate: DAB (Sigma-Aldrich)	1:150
OCT3/4 mouse IgG2b (sc-5279, Santa Cruz)	1:100	goat anti-mouse IgG2b - Alexa 647 (A-21242, Invitrogen)	1:250
SOX2 –Alexa 488 rat IgG2a (53-9811-80, ebioscience)	1:150	pre-labeled	N/A
2A peptide rabbit IgG (ABS31, Millipore)	1:1000	donkey anti- rabbit IgG - Cy3 (711-165-152, Jackson Imm. Research)	1:250

RNA isolation and quantitative RT-PCR (qPCR)

RNA was isolated using the NucleoSpin RNA kits (Macherey-Nagel), including a DNase-digestion step, according to the manufacturer's instructions. For xenografts, RNAlater was removed and all pieces of the same xenograft were homogenized in RA1 buffer (Macherey-Nagel) with 1% β-mercaptoethanol using an Ultra-Turrax T8 homogenizer (IKA Labortechnik). An aliquot of the homogenate was used for RNA isolation. Quantitative expression analysis was performed on a Bio-Rad C1000 Thermal Cycler equipped with a CFX96/384 Real-Time System, with the iQ SYBR Green kit (Bio-Rad). Template cDNA was prepared from 1mg of total RNA using the iScript cDNA synthesis kit (Bio-Rad). hARP was used as endogenous control. The primers used were: SOX2-KLF4 forward: 5'-ACTGCCCCTGTCGCACAT-3', reverse: 5'-CATGTCAGACTCGCCAGGTG-3'; E2A-cMyc forward: 5'-GGCTGGAGATGT-TGAGAGCAA-3', reverse: 5'-AAAGGAAATCCAGTGGCGC-3'; Endogenous SOX2 forward: 5'-GGGAAATGGGAGGGGTGCAAAAGAGG-3', reverse: 5'-TTGC-GTGAGTGTGGATGGGATTGGTG-3'; Nanog forward: 5'-TGCAAGACTCTCCAACATC-CT-3', reverse: 5'-ATTGCTATTCTTCGGCCAGTT-3'; hARP forward: 5'-CACCATTGAAATCCTG-AGTGATGT-3', reverse: 5'-TGACCAGCCCAAAGGA-GAAG-3'.

hPSC Scorecard™ assay

Template cDNA was prepared from 1mg RNA using the High-Capacity cDNA Reverse Transcription Kit (Applied Biosystems) according to the manufacturer's protocol and diluted with RNAse-free H_20

and Taqman® Gene Expression Master Mix (Applied Biosystems). 10µl of this mix was added to each well of the 384-well hPSC Scorecard™ plate (Life Technologies). PCR reactions were run on a Viia7 RT-PCR System (Applied Biosystems) using the 'hpsc-scorecard-template-viia-7-384-well' template from the manufacturers website. Results were analyzed with the hPSC Scorecard™ analysis software (ThermoFisher).

Microarray analysis and PluriTest

For analysis of xenograft global gene expression by the TeratoScore web-resource (Avior et al. Stem Cell Reports 2015), cRNA was labeled and hybridized to Affymetrix Human Genome U133 Plus 2.0 arrays (Dutch Genomics Service & Support Provider, University of Amsterdam UvA, The Netherlands). RNA for analysis with PluriTest and from xenograft samples was labelled and hybridized onto the Illumina Human HT-12 v4 array by GenomeScan B.V., Leiden, The Netherlands. Microarray data was analysed using the R program, version 3.2.2 using the Limma package version 3.26.9. Batch effects were removed using the ComBat function of the sva package version 3.18.0. The PluriTest algorithm is available under www.pluritest.org. The passage numbers of cells analysed by PluriTest are indicated in Table S1.

Statistics

Hierarchical clustering was performed with Euclidian distance and complete linkage. Lists of differentially expressed genes between cell lines/xenografts were derived with a FDR-corrected p-value of 0.05 and $\log_2$-foldchange > 0.5. Results of qPCR and hPSC Scorecard were analysed with SPSS version 23. Statistical significance was determined using the Mann-Whitney U test or Kruskal-Wallis H test, p-values < 0.05 were considered significant.