Transplantation of *Macaca cynomolgus* iPS-derived hematopoietic cells in NSG immunodeficient mice

Soumeya Abed,[1,2,3a] Alisa Tubsuwan,[1,2,4a] Porntip Chaichompoo,[1,2,4] In Hyun Park,[5] Alice Pailleret,[1] Aïssa Benyoucef,[1,6] Lucie Tosca,[7] Edouard De Dreuzy,[1,2,3] Anais Paulard,[9] Marine Granger-Locatelli,[1] Francis Relouzat,[1] Stéphane Prost,[1] Gerard Tachdjian,[7] Suthat Fucharoen,[4] George Q. Daley,[5] Emmanuel Payen,[1,2] Stany Chrétien,[1,2] Philippe Leboulch,[1,8] and Leïla Maouche-Chrétien[1,2]

[1]*CEA, Institute of Emerging Diseases and Innovative Therapies (iMETI), Université Paris Sud 11, France;* [2]*Inserm, Paris, France;* [3]*Université Paris 7, France;* [4]*Thalassemia Research Center, Institute of Molecular Biosciences, Mahidol University, Nakhon Pathom, Department of Biochemistry, Faculty of Medicine Siriraj Hospital and Department of Pathobiology, Faculty of Science, Mahidol University, Bangkok, Thailand;* [5]*Division of Pediatric Hematology/Oncology, Children's Hospital Boston, and Dana-Farber Cancer Institute, Harvard Stem Cell Institute, Harvard Medical School, Boston, MA, USA;* [6]*Inserm U967 - CEA / DSV / IRCM, Fontenay Aux Roses Cedex, France;* [7]*Hôpital Antoine Béclère, Service d'Histologie Embryologie Cytogénétique,"Assistance Publique - Hôpitaux de Paris", Clamart, France;* [8]*Genetics Division, Department of Medicine, Brigham & Women's Hospital and Harvard Medical School, Boston, MA, USA and* [9]*Bluebirdbio, Cambridge, MA, USA*

[a]*These authors contributed equally to this work.*

Correspondence: leila.maouche@inserm.fr
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Supplemental data

Methods

Animals

Two baby female cynomolgus monkeys (three months old) underwent skin punch biopsies and bone marrow (BM) aspiration from the iliac crest. Transplantation experiments were performed in eight-week-old sublethally (3 Gy) irradiated NSG (Nonobese diabetic/severe combined immunodeficient IL-2 receptor γ-chain-null (NOD.Cγ- *Prkdc*^{scid} *Il2r*γ^{tm1Wjl}/SzJ)) immunodeficient mice. All protocols involving animals were approved by the local animal ethics advisory committee, registered with the French Research Ministry and carried out in accordance with French national regulations (based on European directive 2010/63/CE).

Derivation of primary cells, retrovirus production and cynomolgus iPSC derivation

Total BM cells were cultured in α-MEM (PAA) supplemented with 15% heat-inactivated fetal bovine serum (iFBS) from Sera Lab. The medium was replaced after two days and only adherent cells were retained. Three weeks later, cells were phenotyped and identified as mesenchymal stem cell (MSC)-like cells with CD73, CD90 and CD105 antibodies. Fibroblast cells were obtained, by cutting each 4 mm skin biopsy specimen into small pieces in alpha-MEM medium supplemented with 15% iFBS. The skin fragments were held at the bottom of the well with a sterile coverslip, to prevent them from floating and to facilitate fibroblast growth. At confluence, the cells were detached by trypsin treatment and expanded. Only cells from low-number passages were used for iPSC derivation.

Pluripotency was induced with "homemade" VSV-G pseudotyped retrovectors produced in 293-T cells with four MIG plasmids (*hOCT3/4*, *mSOX2*, *mKLF4*, MIG-*hMYC),* each fused to *IRES-GFP*. A low MOI was obtained (10^7 pfu/ml) after concentration. We also purchased high-MOI (10^9 transducing units/ml) retrovectors (Vectalys, France) that did not express GFP.

Briefly, 5 x 10^4 bone marrow mesenchymal or fibroblast cells were plated in each of three wells of a six-well plate, in α-MEM supplemented with 10% iFBS (Life Technologies). The cells were transduced, 24 hours later, with the four vectors, by three different protocols, to determine the best reprogramming protocol for the cynomolgus primary cells. In the first set of conditions, the cells were transduced with *OCT3/4, SOX2* and *KLF4 (O/S/K/M)* retroviruses at an MOI of 10/10/10/5. Four days later, a second round of transduction was performed with O/S/K/M at an MOI of 5/5/5/2.5. In the second set of conditions, the cells were transduced with O/S/K/M at an MOI of 50/50/50/20 and, four days later, a second round of transduction was performed in a similar manner. In the third set of conditions, the cells were transduced with O/S/K/M at an MOI of 100/100/100/20 and, four days later, a second round of transduction was performed at the same MOI. Transductions were performed in the presence of 8 µg/ml protamine sulfate (Sigma- Aldrich). The contents of each well were split into three, 24 hours after the second round of transduction. One of the three resulting wells was treated with 1 mM valproic acid (VPA). Another was treated with a combination of three small molecules (SPT: 2 mM SB431542, 0.5 mM PD0325901 and 0.5 mM thiazovinin). The last well received no chemical treatment. One day later, the cells were detached by treatment with trypsin, counted and plated at a density of 6 x 10^4, 10^5 or 2 x 10^5 cells per 10 cm dish, on irradiated mouse embryonic fibroblasts (MEFs) from the CF1 strain (GlobalStem), in α-MEM supplemented with 10% iFBS, in the presence of the same chemical products (VPA or SPT). The medium was replaced, 24 h later, by standard human ESC medium containing 20% knockout serum (Gibco) and 4 ng/ml FGF2 (Peprotech), as described elsewhere [8]. The medium was then replaced daily, over a period of several weeks. VPA treatment was stopped after seven days, whereas S.P.T levels were maintained in the corresponding wells. Conditioned medium (CM) was added to the transduced primary cells treated with VPA, to compensate for MEF detachment due to VPA toxicity. CM was collected daily from MEFs

incubated for 24 hours in hESC medium. Three to five weeks later, 46 colonies were picked and successfully expanded on MEFs; 31 of these colonies had an indisputable iPSC phenotype (**Supplemental Table S2**). The experimental procedure is summarized **in Supplemental Figure S1**.

Alkaline phosphatase, immunocytochemical staining and teratoma formation

Alkaline phosphatase (AP) staining was performed according to the Millipore AP detection kit instructions. For immunocytochemistry assays, cells were fixed by incubation in 4% paraformaldehyde for 30 minutes, permeabilized in 0.2% Triton X-100 for 30 minutes and blocked by incubation with 3% BSA and 5% donkey serum in PBS (Chemicon). The cells were then incubated with primary antibodies directed against NANOG (1:100) (Abcam ab21624), OCT3/4 (1:100) (Abcam ab19857), SSEA-4 (1:50) (BD Biosciences 560218) and SSEA3-AF488 (1:10) (eBioscience 53-8833). They were incubated with Alexa Fluor 488- or Alexa Fluor 555-conjugated (1:500) donkey anti-rabbit IgG (Life Technologies) as required. For teratoma formation, Cy-iPSCs were treated with 1 mg/ml collagenase IV (Roche), resuspended in a mixture (2:1:1) of DMEM (PAA), Matrigel (Becton Dickinson) and collagen (Life Technologies) and injected intramuscularly into NSG mice. Teratomas formed within 8 to 12 weeks and were excised and fixed. Histological analysis was performed on sections of stained with hematoxylin-eosin.

Reverse transcription-polymerase chain reaction (RT-PCR), quantitative real-time PCR and genomic PCR

Total RNA was extracted from bulk cells, including MEFs, with the PureLink™ RNA Mini Kit (Life Technologies). The RNA was treated with DNase I and subjected to reverse transcription with SuperScript™ III (Invitrogen). Genomic DNA was isolated with the

NucleoSpin® Blood Kit (Macherey-Nagel). Ready-to-use *Taq*Man probes were obtained from Applied Biosystems or synthesized by Eurogentec. Semi-quantitative PCR was performed on a GeneAmp PCR 2400 thermal cycler and quantitative PCR (qPCR) assays were carried out on an ABI PRISM 7300 (Applied Biosystems). Relative fold-changes in expression were calculated by the ΔΔCt method. All the primers and *Taq*man probes used are listed in **Supplemental Table S1.**

Gene methylation profiles and iPSC karyotyping

Two CpG loci in the *Macaca NANOG* promoter, at positions (-700 to -563) and (-558 to -274) relative to the transcription start site (TSS), and in the *OCT3/4* promoter, at positions (-572 to -519) and (-339 to -89) relative to the TSS, were investigated by EpigenDx. Pyrosequencing analysis was performed with the PSQ™96HS system, according to their standard procedures.

Chromosome analysis was performed on cy-iPSCs obtained from the two monkeys, by standard procedures (RHG and GTG banding). For mitotic preparations, cy-iPSCs were cultured in ESC medium supplemented with 0.02 mg/ml colchicine (Eurobio, France) for 2 to 2.5 hours. The cells were detached by trypsin treatment and resuspended in PBS. A warm hypotonic solution of 0.075 M KCl was added and the preparation was incubated for 15 to 20 minutes. Finally, cells were fixed by several incubations in cold Carnoy's fixative (methanol/acetic acid, 3:1).

Hematopoietic differentiation of cy-iPSCs, colony formation in methycellulose and flow cytometry

Undifferentiated cy-iPSC colonies were transferred to ultra-low-attachment dishes (Corning) containing a basic hematopoietic differentiation medium (Basic HDM) for embryoid body (EB) formation. The medium consisted of KO-DMEM (Gibco), 20% FBS (Stemcell

Technologies), 1 mM L-glutamine, 0.1 mM nonessential amino acids (Life Technologies), 0.1 mM β-mercaptoethanol, 50 µg/ml ascorbic acid and 200 µg/ml human holotransferrin (Sigma). The medium was replaced the next day and every three days thereafter, with Basic HDM supplemented with 50 ng/ml hBMP4 and G-CSF, 300 ng/ml hSCF and hFLT3, and 10 ng/ml hIL-3 and hIL-6 (Miltenyi Biotec).

Single-cell suspensions were prepared with an EB dissociation kit according to the manufacturer's protocol (Miltenyi Biotec). CFU assays were performed by plating 100,000 cells on MethoCult® H4434 (Stemcell Technologies). For flow cytometry, cells were reacted with anti-human or specific anti-NHP antibodies against SSEA4, CD31, CD11b (eBioscience), VEGFR2 (R&D), CD144, CD34, CD45, CD105, CD90, CD14, CD73 and CD31 (Becton Dickinson). Live cells were analyzed with a FACS Canto II flow cytometer.

Transplantation into NSG mice

The 17-day-old EBs were dissociated and 10^6 unsorted cells were injected into the right femurs of sublethally irradiated NSG mice. Similar numbers of cells were used for retro-orbital injections. We injected 10^6 freshly sorted cynomolgus BM $CD34^+$ cells, a population enriched in hematopoietic stem cells, into the right femurs of positive control mice. Mice were sacrified 8 to 10 weeks after transplantation. BM cells retrieved from treated and control femurs were analyzed for hematopoietic cell markers, with specific anti-NHP CD45, CD20, CD11b, CD14 and specific anti-mouse CD45 antibodies.

Detection of non-human primate (NHP) DNA in NSG mice and colony-forming cells

For NHP DNA detection in the BM of NSG mice, we performed real-time PCR with probes specific for the MIGR DNA vector and the mouse actin gene. DNA extracted from cy-iPSC

and untreated mouse BM was used to establish standard curves for specific DNA quantification.

Cells retrieved from treated and untreated femurs were plated in methylcellulose H4434 (Stem Cell Technologies) in duplicate, at a density of 1.5 $x10^5$ cells/well. We picked 100 colonies per sample and extracted genomic DNA with the DNA Extract all Reagent kit (Applied Biosystems). We performed qPCR with specific *Macaca* p28S and mouse actin *Taq*Man probes (**Supplemental Table S1),** on an ABI PRISM 7300 (Applied Biosystems).

Figure legends

Supplemental Figure S1

cy-iPSC derivation: Experimental procedure summary for the derivation of iPSCs from cynomolgus MSCs. Two different chemical cocktails were used. The first was a combination of SB431542, PD0325901 and thiazovivin (S.P.T) and the second contained valproic acid (VPA) only. Three different sets of transduction conditions were used, with *hOCT3/4*, *hSOX2, hKLF4* and *hMYC* encoding retrovectors, as indicated. An identical protocol was used for fibroblasts, yielding significantly different results.

Supplemental Figure S2

Characterization of cy-iPSCs. (A): Typical phase-contrast image of an undifferentiated monkey cy-iPS colony growing on MEF feeder cells (left); two representative cy-iPS clones amplified individually in six-well plates and treated with alkaline phosphatase (middle); higher magnification of an alkaline phosphatase-positive colony (right). **(B):** Immunofluorescence assay showing specific staining of one representative cy-iPS clone with antibodies against the pluripotency markers NANOG, OCT3/4 and SSEA-4. **(C):** RT-PCR analysis of the *OCT3/4*, *SOX2*, *NANOG, KLF4, MYC* and *REX1* endogenous (endo) genes in cy-iPSC clones 17, 23, 29 and 36, at low- and high-number passages, and primary MSCs. RT-

PCR on genes expressed from the retroviral vectors encoding the OCT3/4, SOX2, KLF4 and MYC mRNAs (Exo). **(D):** PCR on genomic DNA, showing integration of the four vectors into the genome of cy-iPSC clones 17, 23, 29 and 36. Specific amplification was validated on the negative control (MSCs). **(E):** Histological sections of teratomas formed in NSG mice 8-12 weeks after the injection of cy-iPSCs. Hematoxylin-eosin staining, revealing various tissue derivatives of all three germ layers. **(F)** RT-PCR analysis on cy-iPSC clones 17, 23, 29 and 36, showing the presence of mRNAs for the DNMT1, DNMT3A and DNMT3B methyltransferases at low- and high-number passages. **(G)** Bisulfite genomic sequencing of the *NANOG (left)* and *OCT3/4* (right) promoters of one representative cy-iPSC clone (red line) and the primary MSCs used for iPSC derivation (blue line) revealed a higher proportion of methylated CpGs in the primary cynomolgus MSCs at the indicated positions on the X axis. The positions of the CpGs analyzed are indicated relative to the transcription start site (TSS**)**. **(H)** Karyotypes of cy-iPSC clone 29 (Macaca # 904) and cy-iPSC clone 46 (Macaca # 902), showing normal karyotypes for both (2n=42) at passages 15 and 14, respectively.

Supplemental Figure S3

Hemato/endothelial cell type characterization during EB differentiation kinetic: Expression of the hemato/endothelial CD31, VEGF-R2, CD144, CD34 and CD45 markers, assessed by flow cytometry on single cells prepared from total EBs and their supernatants, on days 2, 4, 5, 7, 10, 14, 18, 21 and 23 of differentiation. The CD144/CD34 analysis (2nd column) was gated on $CD31^{+}VEGF^{-}R2^{+}$ cells (1st column), indicated by the blue arrow.

Supplemental Figure S4

Hematopoietic engraftment of cy-iPSC derivatives in NSG mice: NSG mice underwent transplantation with 10^6 unsorted cy-iPSC-derived cells, obtained from day-17 EBs, into the

right femur (RF). Eight to 10 weeks after transplantation, cells were recovered from the RF and LF, stained with specific NHP-CD45, -CD20, -CD11b, -CD14 and mCD45 antibodies and analyzed by flow cytometry. BM from untreated mice (Ctrl) was subjected to the same antibody staining protocol. The % of NHP-CD45^{+} cells was determined from an mCD45-negative gate. Representative results for right femurs are shown.

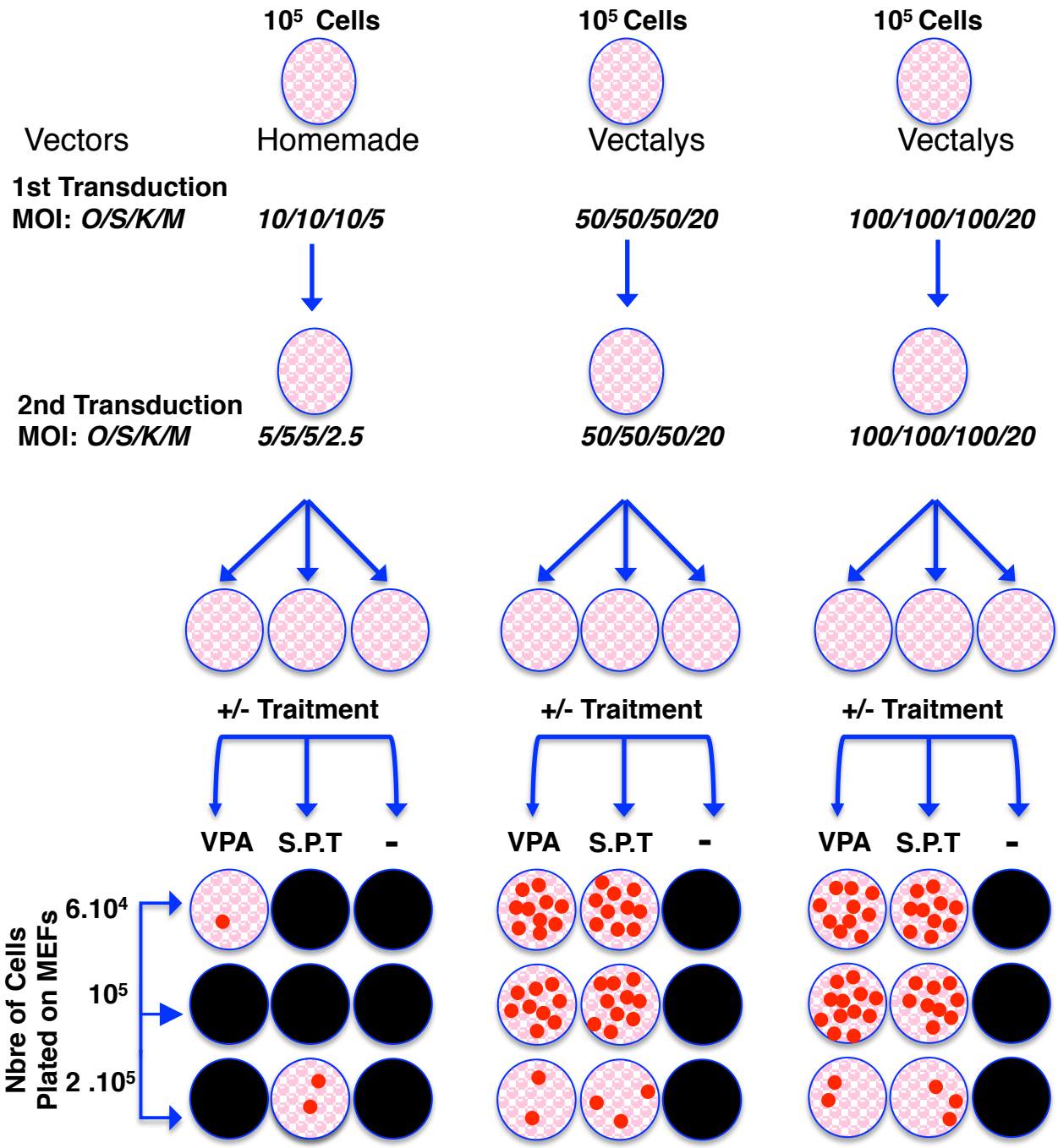
Supplemental Table S1

• Brachyury	• Rh00610080_m1
• CDX4	• Hs00193194_m1
• GATA2	• Rh02850085_m1
• GATA1	• Hs01085823_m1
• ERG	• Hs01554629_m1
• ERG	• Rh02848652_m1
• FLi1	• Hs00956711_m1
• RUNX1	• Hs01021967_m1
• RUNX1	• Rh02839694_m1
• Tie1	• Rh02794015_m1
• Tie1	• Hs00178500_m1
• CEB/P α	• Hs00269972_s1
• GAPDH	• Rh02621745-g1
• TAL1	• Hs00268434_m1
• MYB	• Hs00920554_m1
• PU1	• Hs02786711_m1
• VEGF-R2	• Rh02787868-m1
Endo-Rhmyc-F EndoRh-myc-Rev	5' TGGTACTCCATGAGGAGACACC 3' 5' CGCGTAGTTGTGCTGATGTGTGGAG 3'
Endo-cyKLF4-F Endo-cyKLF4-Rev	5' GATTACGCGGGCTGCGGCAAAAC 3' 5' GTGTAAGGCGAGGTGGTCCGACC 3'
EndoRhSox2-S EndoRhSox2-Rev	5' ATGTCCCAGCACTACCAGAG 3' 5' ACAGCCCTCCCATTTCCC 3'
Endo-cyNanog-S Endo-cyNanog-Rev	5' GTCCCCAAAGCTTGCCTTGCT 3' 5' CCAGTTGTTTTTCTGCCACCTCTT 3'
EndoRhOct-F EndoRhOct-Rev	5' TTGAGTAGTCCCTTAGCAAGCC 3' 5' CTTTCTGCAGAGCTTTGATGTCC 3'
Exo-hOCT4-F	5' CCTCACTTCACTGCACTGTA 3'
Exo-hKLF4-F	5' GATGAACTGACCAGGCACTA 3'
Exo-hSOX2-F	5' CCCAGCAGACTTCACATGT 3'
Exo-hMyc-F	5' AAGAGGACTTGTTGCGGAAA 3'
Exo-hO/hS/hK/hM-Rev	5' TCCTGTCTTTAACAAATTGGACT 3'
hREX-1-F hREX-1-Rev	5' CAGTCCAGCAGGTGTTTGC 3' 5' GCATTCTATGTAACAGTCTGAGA 3'
CyDNMT1-F CyDNMT1-Rev	5' GGGCTGGCCTCTATGGAAGGCTCGAG 3' 5' AATGGCTTTGGCCAGGGGCGGCGGCAC 3'
RhDNMT3A-F RhDNMT3A-Rev	5' CGCTGGGTCATGTGGTTCGGAGA 3' 5' CGCTGTCATGGCACACCGGGAACA 3'
RhDNMT3B-F RhDNMT3B-Rev	5' GGTGGAGGCAGACAGTGGA 3' 5' TGGTACATGGCTTTTCGATAGG 3'

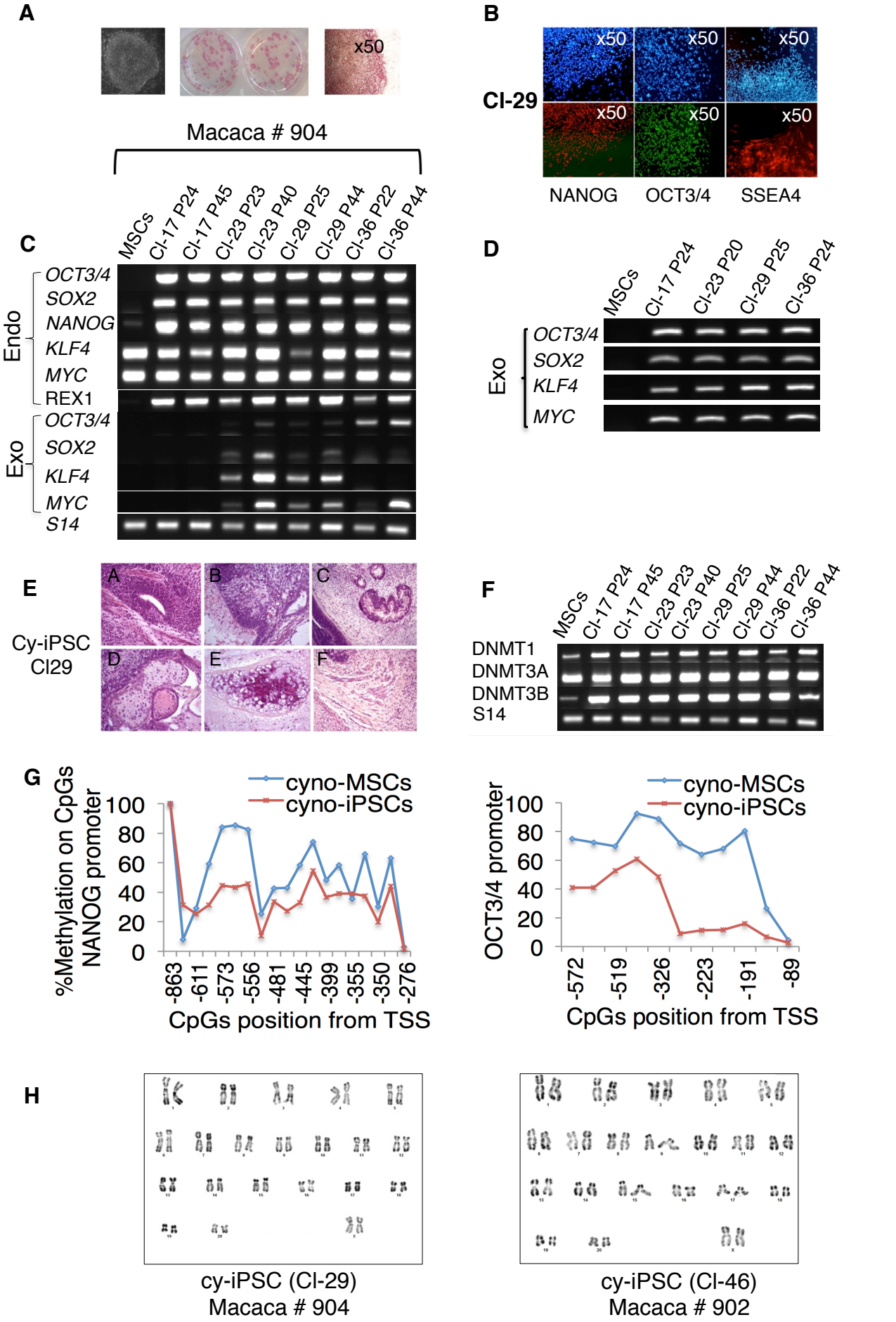
Supplemental Table S2
List of cy-iPSC clones. Their correspondant monkey. The chemical condition used for iPSC derivation. Primary cells used. Vector titers.

Cy-iPSC ID clone	Monkey ID number	Chemical condition	Primary Cells	Vector titer	Clones analyzed
2	*902*	S.T.P	Fibroblasts	Low	+
8	*902*	S.T.P	Fibroblasts	Low	+/-
11	902	VPA	MSCs	Low	+
14	904	S.T.P	MSCs	High	-
15	904	S.T.P	MSCs	High	-
16	904	S.T.P	MSCs	High	-
17	904	S.T.P	MSCs	High	+
19	904	S.T.P	MSCs	High	-
20	904	VPA	MSCs	High	-
21	904	S.T.P	MSCs	High	-
22	904	S.T.P	MSCs	High	-
23	904	S.T.P	MSCs	High	+
24	*902*	S.T.P	MSCs	High	-
25	*902*	VPA	MSCs	High	-
26	904	VPA	MSCs	High	+/-
27	*902*	VPA	MSCs	High	-
28	*902*	VPA	MSCs	High	-
29	904	S.T.P	MSCs	High	+
30	904	S.T.P	MSCs	High	+
31	904	S.T.P	MSCs	High	-
32	904	S.T.P	MSCs	High	-
33	*902*	VPA	Fibroblasts	High	-
34	904	S.T.P	MSCs	High	-
35	904	?	MSCs	High	-
36	904	S.T.P	MSCs	High	+
37	*902*	S.T.P	MSCs	High	-
38	*902*	VPA	MSCs	High	-
39	*902*	VPA	MSCs	High	-
40	904	S.T.P	MSCs	High	-
41	904	S.T.P	MSCs	High	-
46	*902*	VPA	MSCs	High	+

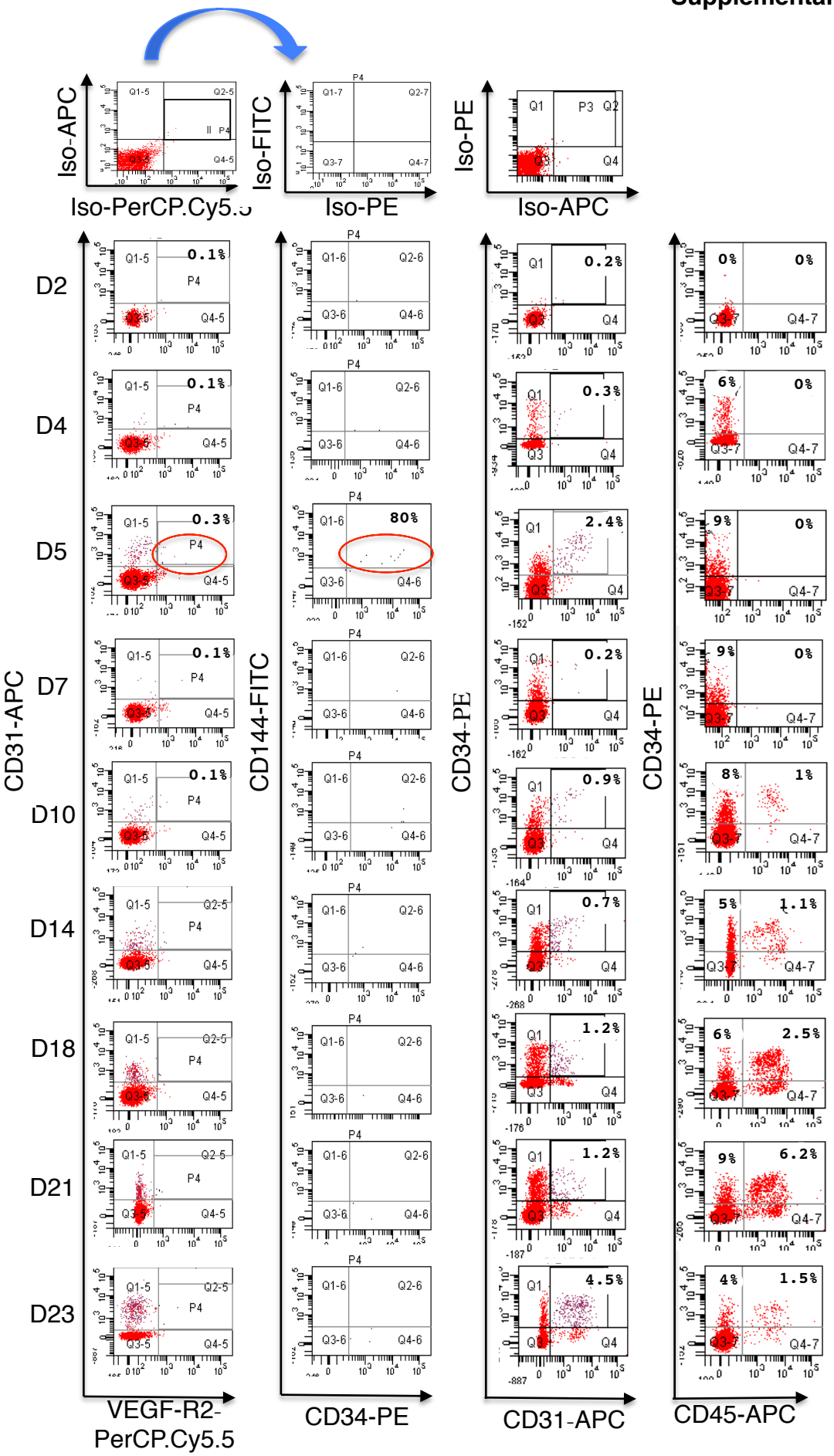
Supplemental Figure. S1



Supplemental Figure.S2



Supplemental Figure.S3



Supplemental Figure. S4