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# Reprogramming human B-cells into induced pluripotent stem cells and its enhancement by C/EBPα

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**Running Title:** iPSC from primary human B-cells and c/EBPα contribution

**Key words:** B cells, iPSC, Sendai virus, OSKM, c/EBPα, Ig gene rearrangements, differentiation potential.

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**ABSTRACT**

B-cells have been shown to be refractory to reprogramming and B-cell-derived induced pluripotent stem cells (iPSC) have only been generated from murine B-cells engineered to carry doxycycline-inducible Oct4, Sox2, Klf4 and Myc (OSKM) cassette in every tissue and from EBV/SV40LT-immortalized lymphoblastoid cell lines. Here, we show for the first time that freshly isolated non-cultured human cord blood (CB)- and peripheral blood (PB)-derived CD19+CD20+ B-cells can be reprogrammed to iPSCs carrying complete VDJH immunoglobulin (Ig) gene monoclonal rearrangements using non-integrative tetracistronic, but not monocistronic, OSKM-expressing Sendai Virus (SeV). Co-expression of c/EBPα with OSKM facilitates iPSC generation from both CB- and PB-derived B-cells. We also demonstrate that myeloid cells are much easier to reprogram than B- and T-lymphocytes. Differentiation potential back into the cell type of their origin of B-cell-, T-cell-, myeloid- and fibroblast-iPSCs is not skewed, suggesting that their differentiation does not seem influenced by "epigenetic memory". Our data reflect the actual cell-autonomous reprogramming capacity of human primary B-cells since biased reprogramming was avoided by using freshly-isolated primary cells, not exposed to cytokine cocktails favoring proliferation, differentiation or survival. The ability to reprogram CB/PB-derived primary human B-cells offers an unprecedented opportunity for studying developmental B-lymphopoiesis and modeling B-cell malignances.

## INTRODUCTION

Induced pluripotent stem cells (iPSCs) provide a unique platform to explore donor/patient-specific somatic cells for regenerative medicine, drug screening and disease modeling[1]. Although the most common source for human iPSC derivation is skin dermal fibroblasts, human iPSCs have been generated from a variety of somatic tissues including keratinocytes[2], mesenchymal stem cells[3] and hematopoietic stem/progenitor cells (HSPC)[4-7]. Generation of iPSC from human hematopoietic cells is an attractive option because they can be generated from peripheral blood (PB) cells which are easily accessible through non-invasive methods and from cord blood (CB) cells which are young cells carrying minimal somatic mutations stored as large collections in public CB banks[8]. To date, human iPSCs have been generated from CD34+ HSPCs[4,6-8] and also from T-cells and myeloid cells[8-10]. However, whether iPSCs may be induced from human normal or leukemic B-cells remains a mystery[6,11]. Reprogramming CB/PB-derived B-cells to pluripotency (iPSCs) will offer a valuable *in vitro* system to study cellular, molecular and epigenetic events underlying the physiology of B-cell lymphopoiesis and the pathogenesis of B-cell malignancies. B-cells have been shown to be refractory to reprogramming[9,10,12], and B-cell-derived iPSCs have only been generated from B-cells of mice engineered to carry doxycycline-inducible Oct4, Sox2, Klf4 and Myc (OSKM) lentiviruses in every tissue[12], and from EBV/SV40LT-transformed lymphoblastoid cell lines[11,13]. Importantly, recent work has revealed that mouse B-cells can be reprogrammed into iPSCs with high efficiency when the cells were pulsed with the CCAAT/enhancer binding protein-$\alpha$ (C/EBP$\alpha$) before of their exposure to the reprogramming factors OSKM[14]. This strongly suggests that biological rather than technical barriers underlie the inability to reprogram B-cells to pluripotency. Here, we show for the first time that freshly isolated non-cultured human B-cells, derived from both CB and PB, can be reprogrammed into iPSCs carrying complete VDJH immunoglobulin (Ig) gene monoclonal rearrangements, using non-integrative polycistronic Sendai Virus (SeV)[15,16]. We also demonstrate that transient co-expression of c/EBPα with OSKM increases B-cell iPSC generation. Differentiation of B-cell-, T-cell-, myeloid- and fibroblast-iPSCs into B-cell, T-cell and myeloid cell fate revealed that iPSC differentiation potential does not seem influenced by the residual “epigenetic memory” of the cell type of origin.

## MATERIAL AND METHODS

### CB and PB collection and isolation of CD19+ and CD19- cell populations

Independent umbilical CB samples (n=10) from healthy newborns and PB samples (n=5) from healthy donors (age 30-40 years old) were obtained from the Barcelona Blood Bank upon approval by our local Ethics and Biozahard Board Committee (ABR/JFJ/S-23). Mononuclear cells (MNCs) were isolated using Ficoll-Hypaque (GE Healthcare, Stockholm, Sweden). After lysing the red blood cells (Lysis solution, Cytognos, Salamanca, Spain), MNCs were stained with anti-human CD19-biotin (eBioscience) and MACS enriched (purity>80%). MACS-enriched CD19+ cells were cultured overnight in RPMI+20%FBS supplemented with hIL7 (10 ng/ml; Preprotech), and then further FACS-purified for CD20 (anti-CD20-APC, BD Biosiences, San Jose, CA) with the FACSAria Fusion flow sorter using "low-recovery high-purity" sorting settings[17]. Post sorting B-cell purity was consistently higher than 99.5% (**Fig 1A**).

### OSKM and C/EBPα Sendai Vector transduction of CB- and PB-derived CD19+ and CD19- cells

Immediately after sorting, between $1x10^5$ and $1x10^6$ fresh, non-stimulated CB/PB-derived CD19+/CD20+ and CB CD19- cells were infected with the tetracistronic defective and persistent Sendai Virus (SeV) encoding *OCT3/4, KLF4, SOX2,* and *c-MYC* factors with the miR-302 target sequence (miROSKM-SeV) (**Fig 1B**) (MOI=3) alone or in combination with the inducible C/EBPαER-SeV (MOI=20) (custom-made from DNAVEC, Tsukuba, Japan) for 3hr at 37ºC. Tetracistronic SeV was developed, generated, reconstituted and tittered as previously described in detail[15,18]. B-cells co-infected with C/EBPαER-SeV, were then half-split and plated onto irradiated mouse embryonic fibroblasts (iMEFs) in RPMI+20%FBS+hIL7, and 24h later β-estradiol (or vehicle) was added to the medium at a final concentration of 100 nM to trigger C/EBPα activity in the nucleus of the infected cells[14]. Then, the cells were changed to hESC medium/MEF-conditioned media (MEF-CM) supplemented with 8ng/mL bFGF (Miltenyi), generated as previously described[19,20]. β-estradiol was maintained for two further days to ensure proper nuclear expression of C/EBPα. This medium was changed every other day and maintained until the iPSC colonies were picked. In order to avoid cell loss during medium changes, floating cells were harvested, centrifuged and plated back on top of the feeders.

Identification of the first emerging hiPSC colonies (~12 days after SeV infection) and passage onto fresh feeders was done mechanically as previously described[6,21]. By day 21, iPSC clones were Tra-1-60 stained to determine the reprogramming efficiency. The generated iPSC clones were maintained on iMEFs in hESC media[6,21] supplemented with 8ng/mL of bFGF. The hESC media was changed every other day, and the cells were passaged using 1 mg/ml collagenase IV every 7-9 days.

**iPSC characterization**

Established iPSCs were fully characterized upon confirmation they were transgene-independent. SeV elimination was determined by qRT-PCR as described[15,18]. Expression of pluripotency markers was performed by immunostaining (alkaline phosphatase, OCT4, NANOG, SOX2 and TRA-1-60) and quantitative RT-PCR (*OCT4, NANOG, SOX2, REX1*) using the antibodies and primers previously described (**Table S1**)[22,23]. G-banding karyotype, *in vivo* teratoma formation and immunocytochemistry for α-smooth muscle actin, FOXA2 and β-III tubulin, was performed as it has been extensively described by our group[21,24,25].

**Bisulfite pyrosequencing of OCT4 and NANOG promoters**

Bisulfite modification of genomic DNA was performed with the EZ DNA Methylation-Gold kit (Zymo Research) following the manufacturer's instructions. The set of primers for PCR amplification and sequencing of *NANOG* and *OCT4* were designed using the software PyroMark Assay Design (version 2.0.01.15; Qiagen): Forward-*NANOG* (5´-TAT TGG GAT TAT AGG GGT GGG TTA-3´), Reverse-*NANOG* (5´-[Btn]- CCC AAC AAC AAA TAC TTC TAA ATT CAC-3´), and sequencing primer S-*NANOG* (5´-ATA GGG GTG GGT TAT-3´); Forward-*OCT4*_prox (5´- GGG GTT AGA GGT TAA GGT TAG TG-3´), Reverse-*OCT4*_prox (5´-[Btn]- ACC CCC CTA ACC CAT CAC-3´), and sequencing primer S-*OCT4*_prox (5´-GGG GTT GAG TAG TTT-3´); Forward-*OCT4*_dist (5´- TTT TTG TGG GGG ATT TGT ATT GA-3´), Reverse-*OCT4*_dist (5´-[Btn]- AAA CTA CTC AAC CCC TCT CT-3´), and sequencing primer S-*OCT4*_dist (5´-ATT

TGT ATT GAG GTT TTG GA-3´)[26]. Primer sequences were designed to hybridize with CpG-free sites to ensure methylation-independent amplification. PCR was performed with primers biotinylated to convert the PCR product to single-stranded DNA templates, using the VacuumPrep Tool. After PCR amplification, pyrosequencing reactions and methylation quantification were performed using PyroMark Q24 reagents, equipment and software, according to manufacturer's instructions.

**TCR and Ig gene monoclonal rearrangements**

In order to determine the myeloid, T-cell or B-cell origin of the established iPSCs, TCR and Ig gene monoclonal rearrangements were analyzed. Genomic DNA from iPSCs and normal PB (polyclonal control) was isolated using standard methods. For Ig gene monoclonal rearrangements, complete VDJH and incomplete DJH rearrangements were amplified and identified using the BIOMED-2 framework 1-3 strategy[27]. For amplification of complete VDJH rearrangements a set of family-specific primers of the FR1 and FR2 regions and one JH consensus primer were used in two multiplexed PCR reactions. Amplification of incomplete DJH rearrangements was performed in two different reactions using family-specific primers for DH1 to DH6 families, respectively, together with the consensus JH primer. The monoclonal nature of the rearrangements was analyzed by the identification of single amplification peaks by GeneScanning analysis following described criteria[28,29]. All products were sequenced as previously described[28]. Monoclonal PCR products were purified with ExoSap™ (USB Co, Cleveland, OH) and directly sequenced in an 3500XL Genetic Analyzer (Applied Biosystems) using BigDye® Terminators with the v1.1 Cycle Sequencing kit (Applied Biosystems)[28]. TCR rearrangements were also amplified and identified using also the BIOMED-2 strategy. TCR gamma (TCRγ) gene rearrangements were analyzed using the TCRγ primer set that identifies the majority of monoclonal T-cell populations[27].

**Myeloid, B-cell, T-cell re-differentiation and spontaneous differentiation assays**

Undifferentiated B-cell-, T-cell-, myeloid- and fibroblast-iPSCs were re-differentiated into myeloid cells, T-cells and B-cells following protocols extensively established. Briefly, for myeloid differentiation confluent iPSCs were transferred into low-attachment plates and allowed to form embryoid bodies (hEBs) in differentiation media (DM) supplemented with hematopoietic cytokines (300ng/ml SCF, 10ng/ml IL-3, 10ng/ml IL-6, and 50ng/ml G-CSF, 25ng/ml BMP-4). At day 15 of hEB development a single cell suspension was achieved using collagenase B followed by 10min treatment with cell dissociation buffer, and by gentle pipetting and stained with anti-CD31-FITC, anti-CD45-APC, anti-CD34-PE and 7AAD (BD Biosciences). Live cells (7AAD-) were analyzed using a FACSCanto-II flow cytometer. Hematopoietic cells were identified as $CD34^{+}CD31^{+}CD45^{-}$ (hemogenic progenitors) and CD45+ cells. The myeloid clonogenic potential was determined by Colony-Forming Unit (CFU) assay by plating 50000 d15 hEB cells into methylcellulose H4435 (Stem Cell Technologies). Cells were incubated at 37°C/5% $CO_2$ humidified atmosphere, and colonies counted after 14 days using standard morphological criteria[17,19,30-34].

For B-cell and T-cell differentiation, hiPSCs were harvested by 40 min collagenase IV/Dispase (Invitrogen) pretreatment and seeded onto over-confluent OP9 stroma on gelatinized 10cm dishes. Culture media consisted of αMEM supplemented with 10% FBS, 1% PenStrep, 100μM monothioglycerol (MTG) and 50 μg/mL ascorbic acid (AA). After 10 days of co-culture onto OP9 stroma, cells were harvested by 30 min digestion with collagenase IV (200U/mL) followed by 15 min with TrypLE (Invitrogen) and CD34+ cells were MACS-purified using CD34 microbeads (Miltenyi) following manufacturer´s instructions. To induce B-cell differentiation, MS5 stroma was used[35]. Isolated iPSC-derived CD34+ cells were co-cultured onto confluent MS5 monolayers at a density of ~$5x10^4$ cells/10cm dish in αMEM supplemented with 10% FBS, 1%PenStrep, IL-7 (20ng/mL), SCF (50 ng/mL) and Flt3L (50ng/mL) (all from R&D Systems). MS5/CD34+ co-cultures were maintained for 30 days and then harvested by collagenase IV/trypLE digestion and stained with CD29-FITC, CD19-PE and CD45-APC (Miltenyi)[35]. To induce T-cell differentiation, purified iPSC-CD34+ cells were co-cultured on confluent monolayers of OP9-DLL4 in αMEM supplemented with 10% FBS, 1%PenStrep, 100μM MTG, 50 μg/mL AA, IL-7 (5 ng/mL), SCF (10 ng/mL) and Flt3L (5 ng/mL). OP9-DLL4/CD34 co-cultures were split every 6-8 days and maintained for 30 days. Co-cultures were then

harvested by collagenases IV/trypLE digestion and stained with CD5-APC and CD45-PerCP-Cy5.5 (BD Biosciences)[36].

For spontaneous differentiation, iPSCs/ESCs were harvested with Collagenase IV (1mg/ml) and cells clumps were transferred into low-attachment plates for EB formation. EBs were maintained for 6 days in DM (KO-DMEM plus 20% KO-serum replacement, 0.1 mM β-mercaptoethanol, 1mM L-glutamine, 1% PenStrep, 1mM sodium pyruvate and 1X non-essential amino acids). Medium was changed every 2 days and EBs were collected after 6 days for RNA extraction and qRT-PCR. Primers used are shown in **Table S1**.

## RESULTS

### Generation of iPSC from CB- and PB-derived human primary B-cells

In ten independent experiments, between $1x10^5$ and $1x10^6$ highly purified (>99.5%) CB-derived CD19+/CD20+ B-cells and CD19- non-B cells were infected with non-integrative tetracistronic SeV encoding OSKM with the miR-302 target sequence (**Fig 1a,b**)[9,15]. Infected cells were cultured on iMEFs in MEF-CM until the emergence of iPSC-like colonies which were plucked and expanded for further 15 days on iMEFs in hESC media into stable iPSC with *bona fide* hESC morphology (**Fig 1c**). From CB, a total of 154 (efficiency: 0.051%) and 33 (efficiency: 0.0013%) iPSC clones were generated from the CD19- and CD19+ cell fractions, respectively (**Table 1**), indicating that the CD19- fraction is reprogrammed into pluripotency with ~40-fold higher efficiency than the CD19+ cell fraction. Lymphocyte development involves sequential DNA genetic rearrangements of the T-cell receptor or Ig loci[37,38]. Thus, to investigate whether the iPSCs were derived from B, T or myeloid (non-B non-T) cells we analyzed both TCR and Ig gene rearrangements using the BIOMED-2 strategy[27]. Three out of the 154 iPSCs generated from the CB-derived CD19- fraction tested positive for TCR Vγ-Jγ rearrangement (**Fig S1a**) while the remaining 151 iPSCs were negative for both TCR and Ig rearrangements (**Fig S1a & Fig 1d**) indicating that 151 iPSCs have originated from myeloid cells (0.05% efficiency) and 3 iPSCs from T cells (0.001% efficiency) (**Table 1**). Upon gradient centrifugation of MNCs and CD19 MACS-depletion, the ratio T-cell:myeloid cell was 1:1 (data not shown), confirming that myeloid cells are much easier to reprogram than T-cells.

Within the CB-derived CD19+ fraction, 27 out of the 33 iPSCs tested positive for complete VDJH Ig monoclonal rearrangement (**Fig 1d,e** & **Table 1**) demonstrating that these iPSCs have originated from terminally differentiated CD19+ B-cells, although at low efficiency, 0.001%. B-cell iPSCs were consistently generated from all the individual CB assayed. The remaining 6 iPSCs tested negative for both Ig and TCR rearrangements. Because FACS purity was >99% (**Fig 1a**), these 6 iPSC lines seem the result of contaminating myeloid cells (**Table 1**). We then characterized several CB-derived B-cell iPSCs (**Fig 1**), T-cell iPSCs (**Fig S1**) and myeloid iPSC (**Fig S2**). They displayed hESC-like morphology (**Fig 1f, Fig S1b, Fig S2a**) and were positive for alkaline phosphatase (**Fig 1f, Fig S1b, Fig S2a**) and NANOG, OCT4, TRA-

1-60 and SOX2 (**Fig 1g, Fig S1c, Fig S2b**). By qRT-PCR, all iPSCs expressed the pluripotency factors *NANOG, OCT4, SOX2* and *REX1* (**Fig 1h, Fig S1d, Fig S2c**), and by passage 10 they have completely eliminated SeV (**Fig 1i, Fig S1e, Fig S2d**), indicating they are *bona fide* transgene-free iPSCs. All iPSCs were kariotypically normal (**Fig 1j, Fig S1f, Fig S2e**), and consistent with the activation of endogenous pluripotency genes, reprogramming was accompanied by an extensive loss of CpG methylation at the *OCT4* and *NANOG* promoters (**Fig 1k, Fig S1g, Fig S2f**).The most rigorous pluripotency test of human iPSC is the formation of teratomas in immunedeficient mice[25]. On subcutaneous injection into NSG mice, all iPSC generated well-differentiated teratomas representing the three germ layers (**Fig 1l, Fig S1h, Fig S2g**).

For PB-derived mature B-cells (n=5), highly purified (>99.5%) CD19+/CD20+ B-cells were reprogrammed using the conditions and miROSKM-SeV as described above for CB B-cells (**Fig 1a,b**). Under basal conditions, 10 iPSC clones were generated resulting in a reprogramming efficiency of 0.001%, very similar to that of CB B-cells (**Table 2** & **Fig 2b**). These PB B-cell iPSCs displayed hESC-like morphology (**Fig 2c**), had complete VDJH Ig monoclonal rearrangement (**Fig 2d**), and expressed (at both RNA and protein level) the pluripotency factors *NANOG, OCT4, SOX2*, *REX1* and Tra-1-60 (**Fig 2e,f**).

**Transient expression of C/EBPα facilitates OSKM-mediated CB and PB B-cell reprogramming**

We recently demonstrated that mouse B-cells can be reprogrammed into iPSCs with high efficiency when the cells were pulsed with the C/EBP$\alpha$ before exposure to the reprogramming factors OSKM[14]. We thus hypothesized that C/EBP$\alpha$ may function as a "path-breaker" facilitating human B-cell reprogramming. CB and PB mature B-cells were reprogrammed basically as described above but they were now co-infected with miROSKM-SeV and C/EBPαER-SeV and then maintained the first 72h in the presence of β-estradiol (or vehicle) to trigger C/EBPα activity in the nucleus of the infected cells[14] (**Fig 2a**). C/EBPα pulse increased ~2-fold the efficiency of OSKM-mediated PB- B-cell reprogramming (**Fig 2b** & **Table 2**). C/EBPα-mediated PB B-cell iPSCs (Bα-cell iPSCs) were characterized for morphology (**Fig 2c**), complete

VDJH Ig monoclonal rearrangement (**Fig 2d**), and RNA and protein expression of the pluripotency factors *NANOG, OCT4, SOX2*, *REX1* and Tra-1-60 (**Fig 2e,f**), resulting indistinguishable from those B-cell iPSCs generated in the absence of C/EBPα. Similarly, co-expression of C/EBPα and OSKM also enhanced ~2.5-fold the reprogramming efficiency of B-cells from CB (**Fig 2g**). CB-derived B-cell iPSCs and Bα-cell iPSCs were also morphological, molecular and phenotypically indistinguible (**Fig 2h-j**). Addition of sodium butyrate (NaB), an HDAC inhibitor previously reported to facilitate iPSC generation[39] did not display an effect on OSKM-mediated B-cell reprogramming when used either alone or in combination with C/EBPα (**Table 2**).

**The differentiation potential of B-cell-, T-cell- and myeloid-iPSCs is not influenced by the cell type of origin**

There is controversy on whether iPSCs retain residual 'epigenetic memory' of the donor tissue/cell type from which they were derived and display skewed differentiation potential[40]. Here, we next tested and quantified whether the differentiation potential of B-cell-, T-cell-, myeloid- and fibroblast-iPSCs is determined by the cell type of origin. Myeloid differentiation from the indicated B-cell, T-cell, myeloid- and fibro-iPSCs (n=3) was assessed by FACS (CD45+CD33+) and CFU potential using a hEB formation system[17,19,30-34] (**Fig 3a**). As compared with B-cell-, T-cell- and fibroblast-iPSCs, myeloid-iPSCs displayed a slight trend towards the generation of more hematopoietic cells (p-value>0.1; **Fig 2b**) with higher CFU potential (p-value>0.1; **Fig 2c**). Among all the different iPSCs tested, the levels of B-cell (CD45+CD19+) differentiation on MS5-iPSC co-culture (**Fig 2d,e**) and T-cell (CD45+CD5+) differentiation on OP9-DLL4-iPSC co-culture (**Fig 2d,f**) ranged between 0.2%-1% and 20%-60%, respectively, with no trend of predisposed differentiation ("epigenetic memory") towards the cell type from which they were derived. Next, we evaluated more globally the developmental potential of the different B-cell-, Bα-cell-, T-cell-, myeloid-iPSCs. Upon hEB spontaneous differentiation, qRT-PCR was performed for mesoderm- (CXCR4, PDGRFα, PDGFRβ), ectoderm- (MAP2, PAX6) and endoderm-specific (SOX17, AFP, FOXA2) master factors (**Fig S3a**). All iPSCs readily differentiated into tissues representing the three germ layers with no evident difference depending the cell type from which the iPSCs were derived (**Fig S3b**). Altogether, these

data indicate that re-differentiation back into the cell type of origin of B-cell-, T-cell-, myeloid- and fibroblast-iPSCs is not skewed, suggesting that their differentiation potential does not seem influenced by "epigenetic memory".

**DISCUSSION**

To date, human primary B-cells have been shown to be refractory to reprogramming[9,10,12]. Here, we show for the first time that human CB/PB-derived B-lymphocytes can be induced to pluripotency using a non-integrative tetracistronic miROSKM-SeV, and that the B-cell reprogramming process is enhanced by co-expression of OSKM with the "path breaker" cEBPα. Our systematic study using CB and PB demonstrates that miROSKM-SeV allows the generation of iPSC from primary myeloid, T-cell and B-cells. Myeloid cells are the easiest to reprogram in contrast with a previous report describing higher reprogramming efficiency of pre-stimulated T-lymphocytes as compared to myeloid cells[10]. Our data reflect the actual intrinsic cell-autonomous reprogramming capacity of myeloid, B-cells and T-cells since we have avoided unbiased or skewed reprogramming by using freshly-isolated uncultured primary cells, not exposed to hematopoietic cytokines favoring proliferation or differentiation. The efficiency of SeV infection was similar (~85%) in CD19+ and CD19- cells, indicating that SeV-mediated reprogramming efficiency is not associated to viral infection efficiency. Importantly, however, the tetracistronic miROSKM-SeV system was compared side-by-side with the monocistronic SeV system (four genes in four different plasmids). Only B-cell iPSCs were obtained when the tetracistronic miROSKM-SeV was used. In contrast, reprogramming efficiency of myeloid cells was similar upon delivering the OSKM factors in one or several vectors (data not shown), confirming that the delivery of all the reprogramming factors in one genome may be a requirement for B-cell reprogramming, likely by increasing the likelihood of simultaneous expression of the four factors within the same cell.

We found efficiencies of iPSC induction for myeloid and T-cells similar to previous reports[7,9,10]. Chou BK *et al*[41] found that by using an episomal reprogramming plasmid the efficiency of iPSC generation is higher in CB than in PB. However, using miROSKM-SeV we here report that CB- and PB-derived B-cells are reprogrammed at very similar efficiency, likely reflecting that potential reprogramming barriers such as more mature phenotype and lower proliferation index of activated circulating B-lymphocytes may be circumvented with the use of a robust tetracistronic miROSKM-SeV. Despite successful reprogramming of CB/PB-derived B-cells, the efficiency remains very low. Reprogramming involves a concomitant silencing of the

transcriptional program specific of the somatic cell and activation of a pluripotent transcriptome whereas mature B-cells depend upon survival signals delivered to the cells by their antigen receptor (BCR)[42]. Thus, if the Ig/BCR locus rapidly switches off upon ectopic OSKM expression then the B-cells will probably not survive long enough to allow for the functional-phenotypic conversion to pluripotency to occur, probably representing a major barrier in B-cell reprogramming.

C/EBPα is a master myeloid transcription factor which induces transdifferentiation of B-cells into macrophages[43]. Earlier work in mouse showed that co-expression of C/EBPα with OSKM increases between 15 and 100-fold the reprogramming efficiency of murine B-cells[12,14]. We reasoned that C/EBPα may also function as a "path-breaker" in human B-cells, thus facilitating the reprogramming of human primary B-cells. We demonstrated that C/EBPα increased 2.5-fold the reprogramming efficiency of both CB and PB B-cells. The C/EBPα-mediated enhancement of B-cell iPSC generation is not as strong as that observed with mouse B-cells[12,14], likely reflecting species-specific differences in B-cell developmental stages. However, technical differences cannot be ruled out because due to the SeV biology target cells have to be simultaneously infected with both miROSKM-SeV and C/EBPαER-SeV, and the miROSKM-SeV used here was not inducible thus preventing us to perform an initial C/EBPα priming followed by OKSM induction. C/EBPα-mediated CB/PB B-cell iPSCs were characterized and resulted indistinguishable from those B-cell iPSCs generated in the absence of C/EBPα. Whether C/EBPα poises human B-cells for rapid reprogramming by inducing the expression of the dioxygenase TET2 which, in turn, makes the chromatin of pluripotency genes more accessible by promoting de-methylation of such genes by conversion of 5mC to 5hmC requires further investigation.

It has been suggested that iPSCs may retain a residual 'epigenetic memory' of the donor tissue/cell type from which they are derived, and therefore the donor cell type can influence the epigenome and differentiation potential of hiPSCs[40,44]. However, almost all of these studies have compared the differentiation potential of iPSCs developed from tissues/cell types with different developmental origin.

Here, we analyzed whether the differentiation potential of iPSCs derived from distinct blood cell types (B-cells, T-cells and myeloid cells) from the same tissue (CB) and same donors is determined by the cell type of origin. Using robust direct differentiation protocols[30,32,35,36], B-cell-, T-cell- and myeloid-iPSCs were re-differentiated back into myeloid cells, B-cells and T-cells. In addition, more global developmental potential into mesoderm-, ectoderm- and endoderm was also assessed upon spontaneous hEB differentiation. Overall, no 'epigenetic memory'/predisposed differentiation towards the cell type of their origin was found, indicating that the developmental differences between CB-derived myeloid, B-cells and T-cells are not instructive enough as to influence the differentiation potential of the resulting hiPSCs. Residual “epigenetic memory” is thought to be progressively lost upon cell passaging. In this study, all the iPSCs used for differentiation assays were at passage 20-30, when no traces of miROSKM-SeV were detected, and thus some degree of predisposed differentiation/”epigenetic memory” at early-passage cannot be ruled out. In addition, it should be taken into consideration that a limited number of iPSC lines from distinct healthy individuals were used in this study and therefore these data must be validated in a larger cohort of blood cell type-derived iPSCs from a larger variety of donors.

In sum, generation of iPSC from human hematopoietic cells from PB and CB is an attractive option because they are easily accessible through non-invasive methods, and in the case of CB, cells are young and harbor minimal somatic mutations. Reprogramming healthy donor and/or patient-specific PB-derived B-cells to pluripotency will offer a valuable *in vitro* system to study cellular, molecular and epigenetic events underlying the physiology of B-cell lymphopoiesis and the pathogenesis of B-cell malignancies. This study should encourage further strategies to increase the reprogramming efficiency of normal and leukemic B-cells for downstream applications such as disease modeling, drug screening and developmental immunology.

**AUTHOR CONTRIBUTION**

C.B, J.L.S: conceived the study, designed and performed experiments, analyzed data and wrote the manuscript. K.N, M.O, M.N: contributed key reagents and interpreted the data. B.DS, D.R-M, A.M-L, M.C.C, A.B, L.A, J.C, A.H, O.Q-B, J.C.S, M.F.F, A.F, I.G: performed experiments. T.G: conceived the study. P.M: conceived the study, designed experiments, analyzed data and wrote the manuscript.

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**CONFLICT OF INTEREST:** The authors have no conflict of interest to disclose. All authors have read and approved the manuscript in its present form.



## Supplementary information is available at Leukemia's website.

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**LEGEND TO FIGURE 1: Generation and characterization of mature B-cell iPSCs. (a)** Representative flow cytometry plots of CD19+ and CD19- populations before and after FACS sorting (n=15). 99.6% of sorted B-cells were CD19+CD20+ (not shown). **(b)** Genome structure of the OSKM-miR302-expressing SeV vector. **(c)** Schematic drawing of the strategy used to derive iPSCs from B-cells. **(d)** Complete VDJH monoclonal rearrangements were present in all CB-derived B-cell iPSCs. Top panel shows polyclonal complete VDJH rearrangements from normal CB. Bottom panel shows a complete VDJH monoclonal rearrangement in a representative CB-derived B-cell iPSC. **(e)** Representative electropherogram of a B-cell iPSC showing the junction region of the PCR product sequence shown in (D) confirming a complete VDJH monoclonal rearrangement (IGHV1-69*01/IGHD3-9*01/IGHJ4*02). **(f)** Morphology and alkaline phosphatase staining of a representative B-cell iPSC. **(g)** Immunostaining for the pluripotency markers OCT4, NANOG, SOX2 and TRA-1-60 in a representative B-cell iPSCs. **(h)** Q-RT-PCR for the pluripotency transcription factors *OCT4, SOX2, NANOG* and *REX1*. **(i)** Representative Q-RT-PCR demonstrating SeV elimination after 10 passages. The inset is a representative anti-SeV immunostaining showing a high infection (~85%) with SeV of CD19+ B-cells 2 days after infection. **(j)** Diploid karyotype of a representative B-cell iPSC at p15. **(k)** Pyrosequencing revealing demethylation of *OCT4* and *NANOG* promoters in representative B-cell and fibroblast-PSCs. **(l)** Teratoma analysis revealing three germ layer differentiation of a representative B-cell iPSC. α-sm actin, FoxA2 and β-III tubulin are mesoderm-, endoderm- and ectoderm-specific markers.

**LEGEND TO FIGURE 2: C/EBPα expression facilitates OSKM-mediated CB and PB B-cell reprogramming. (a)** Schematic overview of the strategy used to derive iPSCs from CB and PB B-cells in the presence of C/EBP$\alpha$. **(b)** C/EBPα expression induces a 2.5-fold increase in iPSC generation (measured as Tra-1-16+ colonies) from PB B-cells. Data are shown relative to colony number in the absence of C/EBP$\alpha$. Error bars indicate s.d. (n=3). **(c)** Representative morphology of PB B-cell iPSCs and PB B$\alpha$-cell iPSCs. **(d)** Complete VDJH monoclonal rearrangements were present in both PB B-cell and PB B$\alpha$-cell derived iPSCs. **(e)** Representative immunostaining for the pluripotency markers OCT4-SOX2 and TRA-1-60-NANOG in PB B-cell iPSC and PB B$\alpha$-cell iPSC. **(f)** q-RT-PCR for the pluripotency

transcription factors *OCT4, SOX2, NANOG* and *REX1* in PB B-cell iPSC and PB Bα-cell iPSC. PB-MNCs were used as negative controls. Error bars indicate s.d. (n=3). **(g)** C/EBPα expression induces a 2.5-fold increase in iPSC generation (measured as Tra-1-60+ colonies) from CB B-cells. Data are shown relative to colony number in the absence of C/EBPα. Error bars indicate s.d. (n=3). **(h)** q-RT-PCR showing the exogenous expression of C/EBPα in B cells upon infection with the C/EBPαER-SeV. **(i)** q-RT-PCR for the pluripotency transcription factors *OCT4, SOX2, NANOG* and *REX1* in CB Bα-cell iPSC. CB-MNCs were used as negative controls. Error bars indicate s.d. (n=3). **(j)** Representative morphology and immunostaining for the pluripotency markers OCT4, NANOG, SOX2 and TRA-1-60 in a CB Bα-cell iPSCs.

**LEGEND TO FIGURE 3: Myeloid, B-cell and T-cell differentiation of B-cell, T-cell, myeloid- and fibroblast-iPSCs. (a)** Schema of hiPSC hematopoietic differentiation system based on hEB formation and time points for FACS and CFU analysis. **(b)** Hematopoietic potential of the different iPSCs indicated. Inset is a representative FACS analysis of hematopoietic cells (black dots). HEP stands for Hemato-Endothelial Progenitors. Error bars indicate s.d. (n=3). **(c)** Myeloid potential measured as clonogenic capacity of the different iPSCs indicated. Error bars indicate s.d. (n=3). Right panels show representative CFU colonies (top) and flow cytometry (bottom) of the CFUs confirming the myeloid phenotype (CD33+). **(d)** Schema of hiPSC B-cell (OP9 + MS5 co-cultures) and T-cell (OP9 + OP9-DLL4 co-cultures) differentiation systems and time points for FACS analysis. **(e)** B-cell potential measured by FACS (CD45+CD19+) of the different iPSCs indicated. Error bars indicate s.d. (n=3). **(f)** T-cell potential measured by FACS (CD45+CD5+) of the different iPSCs indicated. Error bars indicate s.d. (n=3).

**Table 1:** iPSC induction efficiency from Cord Blood-derived B-cells, T-cells and myeloid cells.

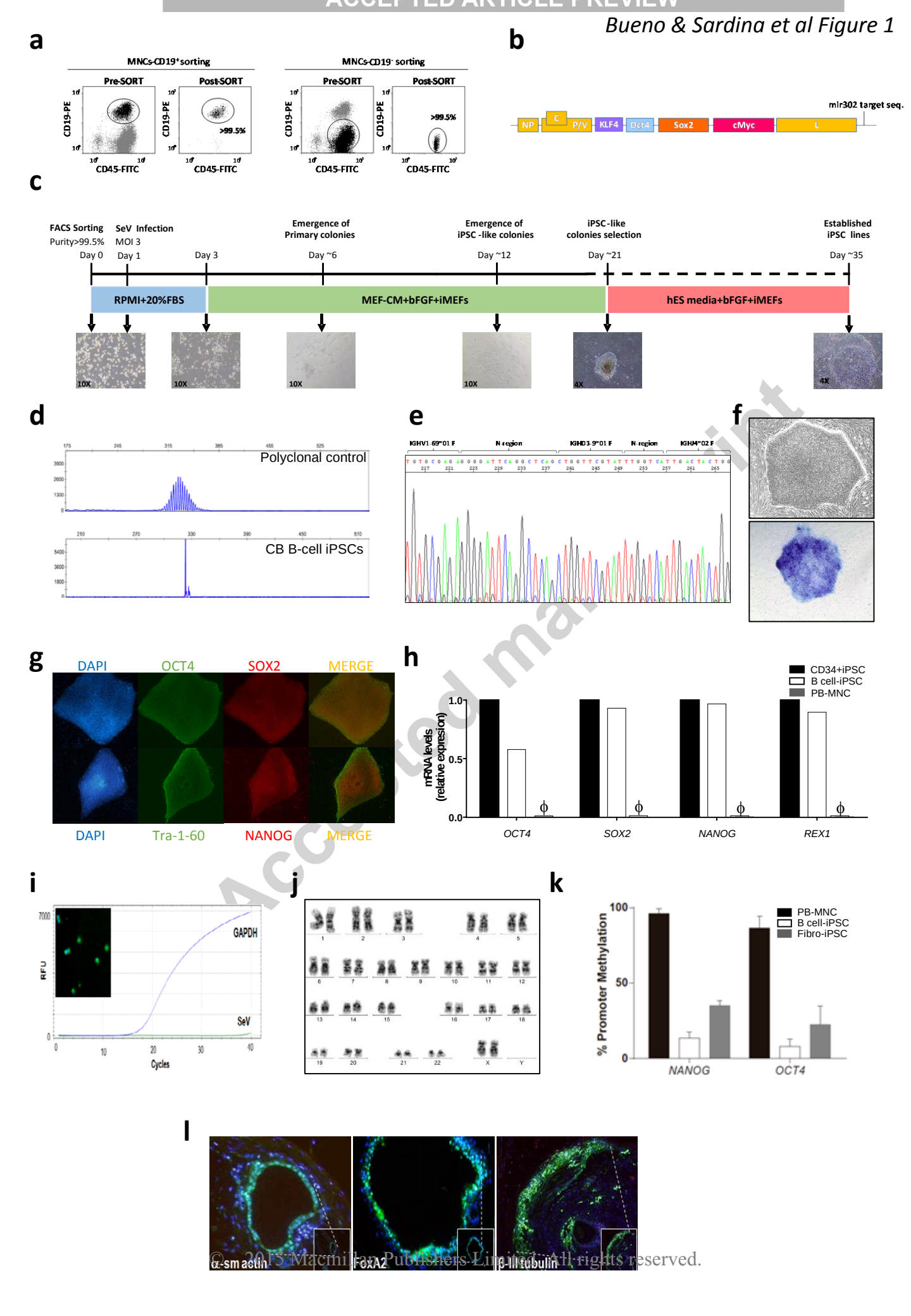
| | Cord Blood (n=10) | |
|---|---|---|
| Cell subset reprogrammed | CD19+ | CD19- |
| B-cell iPSC lines | 27/3.500.000<br>0.001% | 0/300.000<br>0% |
| T-cell iPSC lines | 0/2.000.000<br>0% | 3/300.000<br>0.001% |
| Myeloid iPSC lines* | 6/2.000.000<br>0.0003% | 151/300.000<br>0.05% |

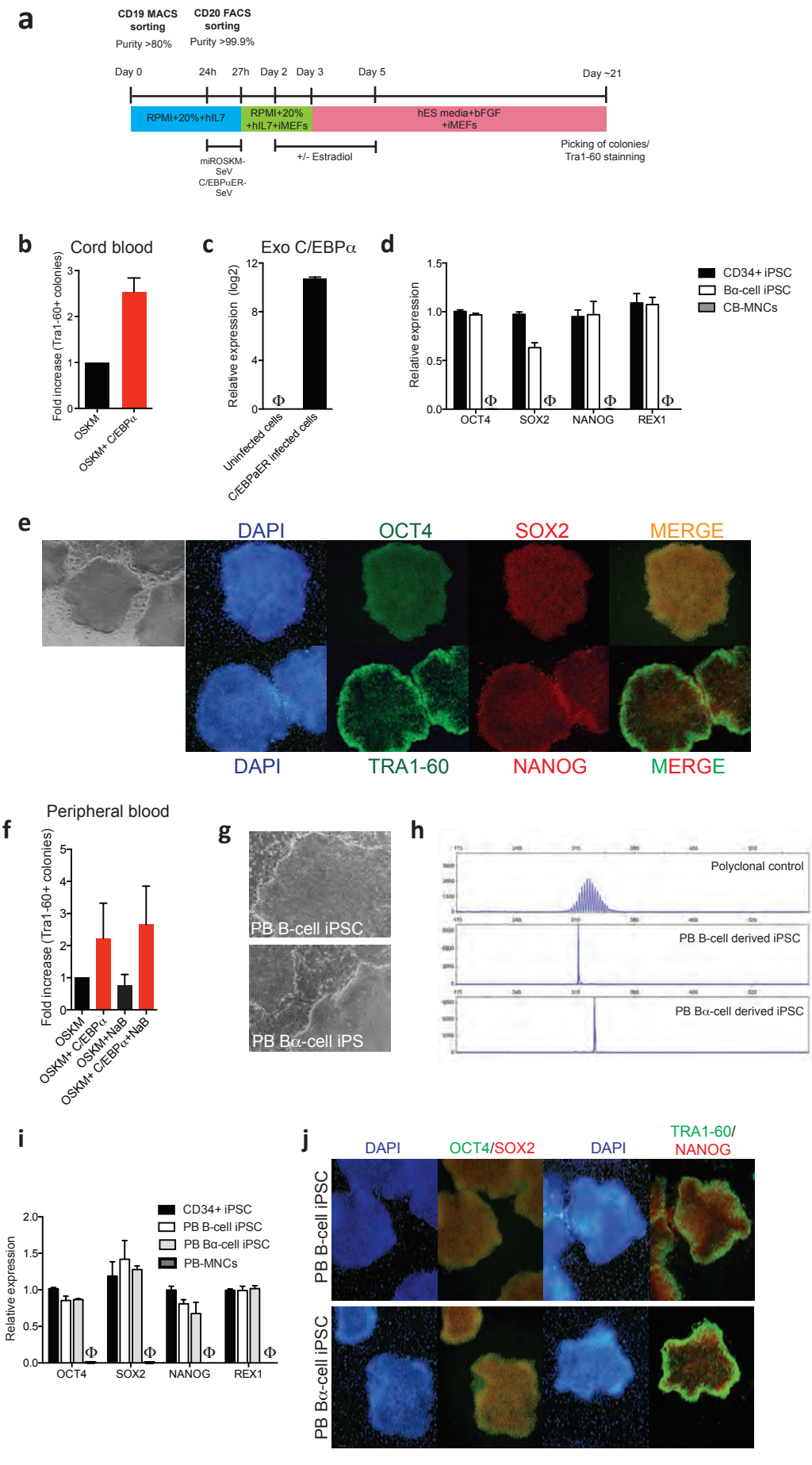
*Indicates non-T, non-B iPSC lacking both TCR and IGH rearrangements.
Data shown as number of iPSC clones/infected cells and reprogramming efficiency (in %).
Identity of B-cell iPSCs was always confirmed by VDJH rearrangements.

**Table 2:** iPSC induction efficiency from Peripheral Blood-derived B-cells in the presence or absence of cEBPα.

| | PB B-cell (n=5) | | | |
|---|---|---|---|---|
| Reprogramming conditions | - | NaB | cEBPα | cEBPα+NaB |
| Efficiency | 10/1.000.000<br>0.001% | 10/1.000.000<br>0.001% | 19/1.000.000<br>0.0019% | 22/1.000.000<br>0.0022% |

Data shown as number of iPSC clones/infected cells and reprogramming efficiency (in %).
Identity of B-cell iPSCs was always confirmed by complete VDJH rearrangements.
NaB: Sodium Butyrate




a
CD19 MACS sorting
Purity >80%
CD20 FACS sorting
Purity >99.9%
Day 0
24h
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Day 2
Day 3
Day 5
Day ~21
RPMI+20%+hIL7
RPMI+20% +hIL7+iMEFs
hES media+bFGF +iMEFs
miROSKM-SeV
C/EBPαER-SeV
+/- Estradiol
Picking of colonies/ Tra1-60 stainning
b
Cord blood
Fold increase (Tra1-60+ colonies)
OSKM
OSKM+ C/EBPα
c
Exo C/EBPα
Relative expression (log2)
Uninfected cells
C/EBPαER infected cells
d
Relative expression
OCT4
SOX2
NANOG
REX1
CD34+ iPSC
Bα-cell iPSC
CB-MNCs
e
DAPI
OCT4
SOX2
MERGE
DAPI
TRA1-60
NANOG
MERGE
f
Peripheral blood
Fold increase (Tra1-60+ colonies)
OSKM
OSKM+ C/EBPα
OSKM+NaB
OSKM+ C/EBPα+NaB
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Polyclonal control
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PB Bα-cell derived iPSC
i
Relative expression
CD34+ iPSC
PB B-cell iPSC
PB Bα-cell iPSC
PB-MNCs
OCT4
SOX2
NANOG
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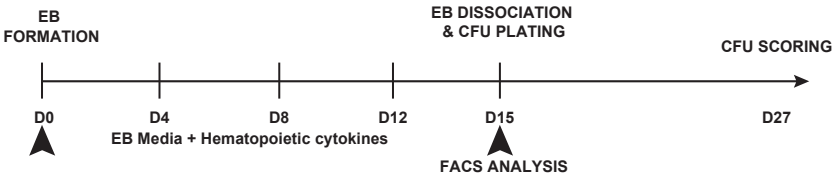
j
DAPI
OCT4/SOX2
DAPI
TRA1-60/ NANOG
PB B-cell iPSC
PB Bα-cell iPSC

**a**

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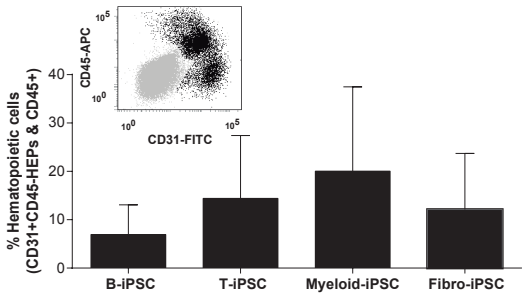


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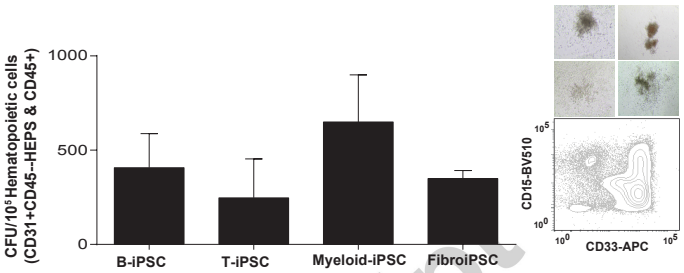


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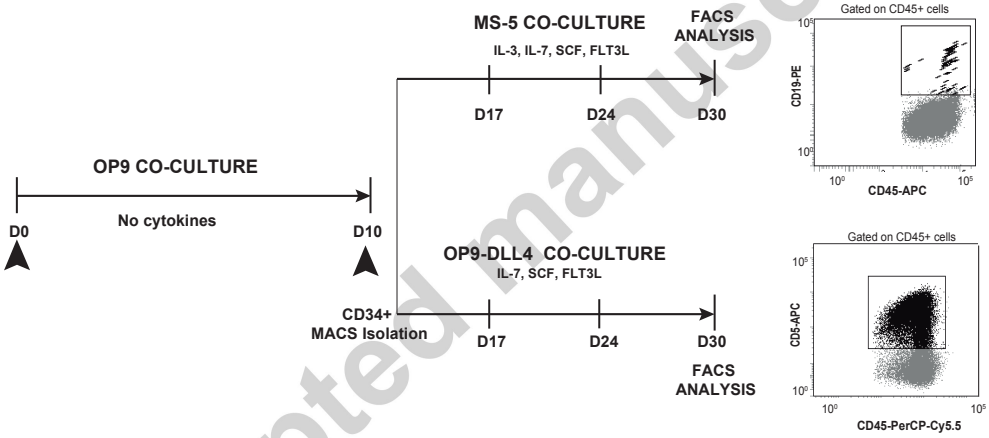


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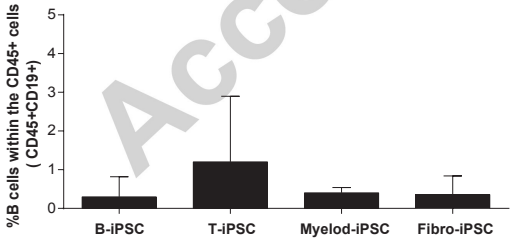


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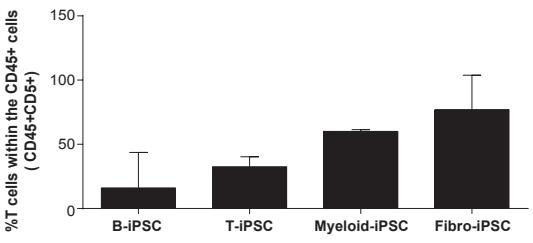
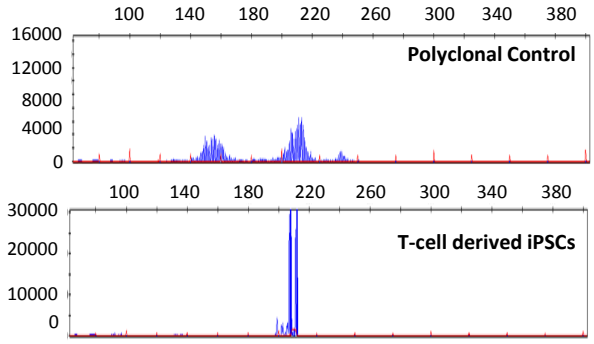


**a**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Polyclonal Control
100 140 180 220 260 300 340 380
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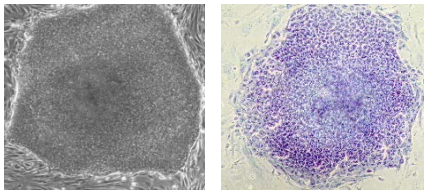
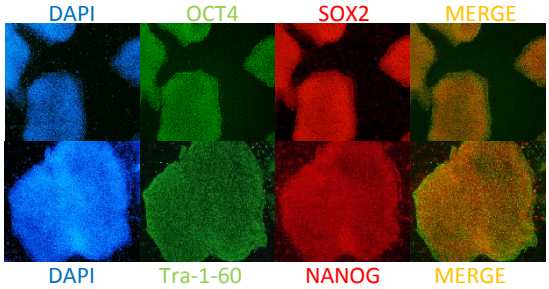
T-cell derived iPSCs

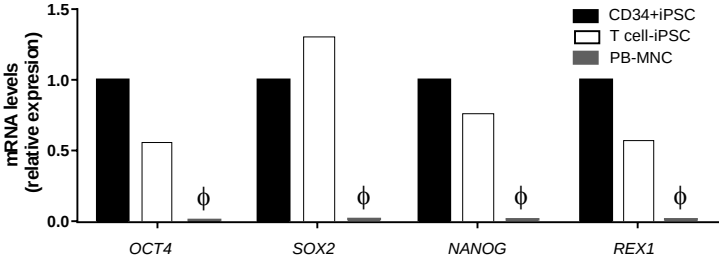
**b**



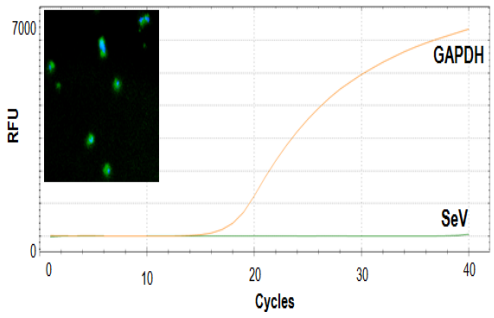
**c**


DAPI
OCT4
SOX2
MERGE
DAPI
Tra-1-60
NANOG
MERGE

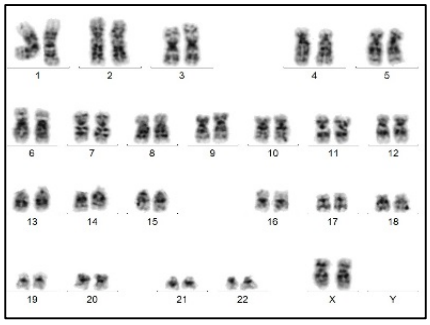
**d**


mRNA levels (relative expresion)
1.5
1.0
0.5
0.0
CD34+iPSC
T cell-iPSC
PB-MNC
ϕ
OCT4
SOX2
NANOG
REX1

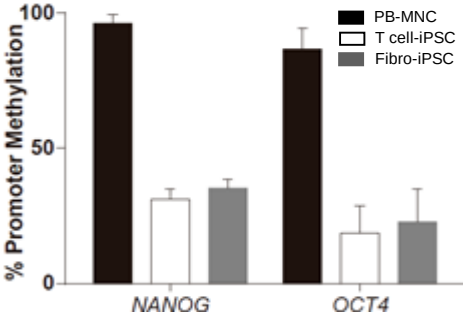
**e**


7000
RFU
0
GAPDH
SeV
0
10
20
30
40
Cycles

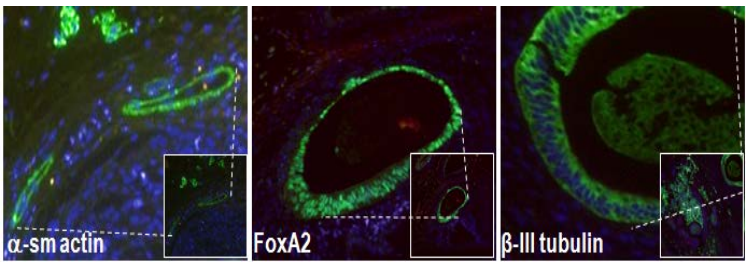
**f**



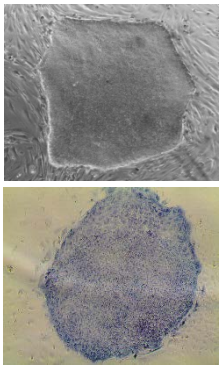
**g**


100
50
0
% Promoter Methylation
PB-MNC
T cell-iPSC
Fibro-iPSC
NANOG
OCT4

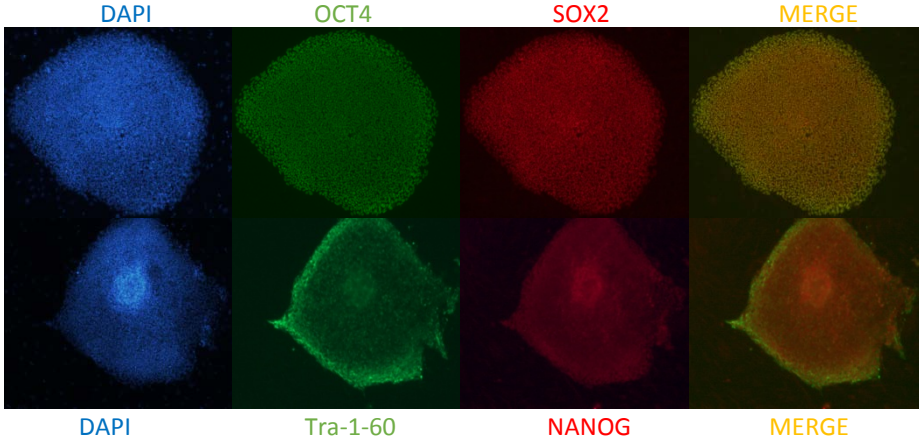
**h**


α-sm actin
FoxA2
β-III tubulin

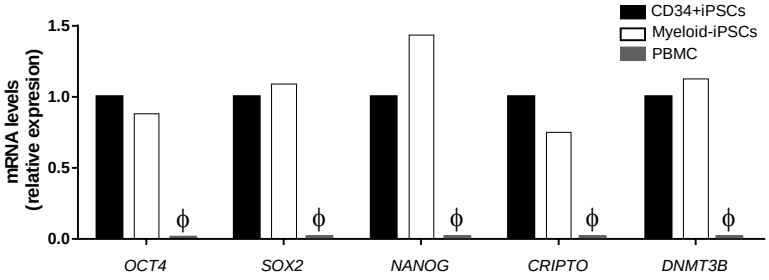
**a**



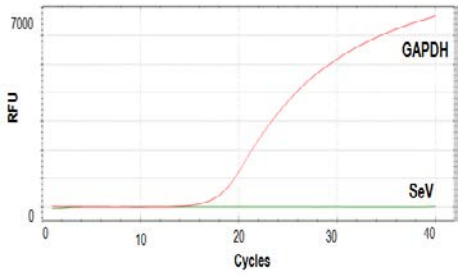
**b**


DAPI
OCT4
SOX2
MERGE
DAPI
Tra-1-60
NANOG
MERGE

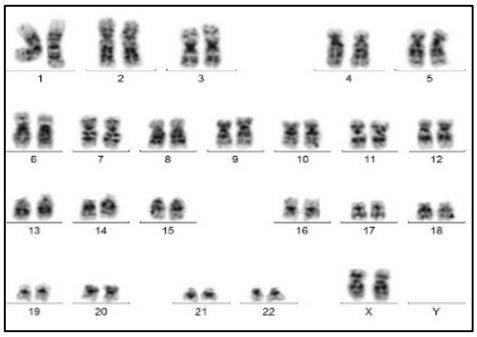
**c**


CD34+iPSCs
Myeloid-iPSCs
PBMC
mRNA levels (relative expresion)
1.5
1.0
0.5
0.0
ϕ
OCT4
SOX2
NANOG
CRIPTO
DNMT3B

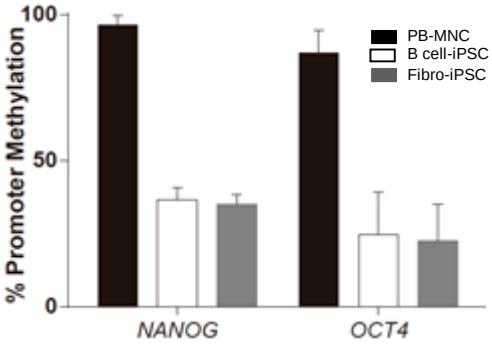
**d**


7000
RFU
0
GAPDH
SeV
0
10
20
30
40
Cycles

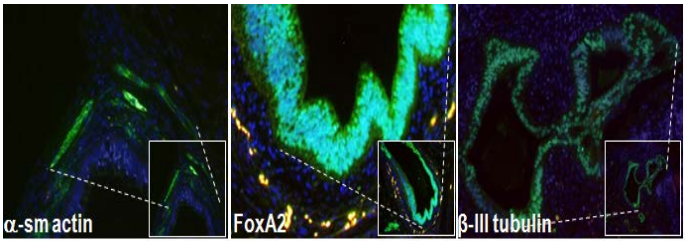
**e**


1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
16
17
18
19
20
21
22
X
Y

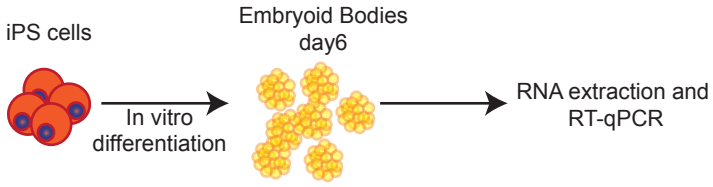
**f**


100
50
0
% Promoter Methylation
PB-MNC
B cell-iPSC
Fibro-iPSC
NANOG
OCT4

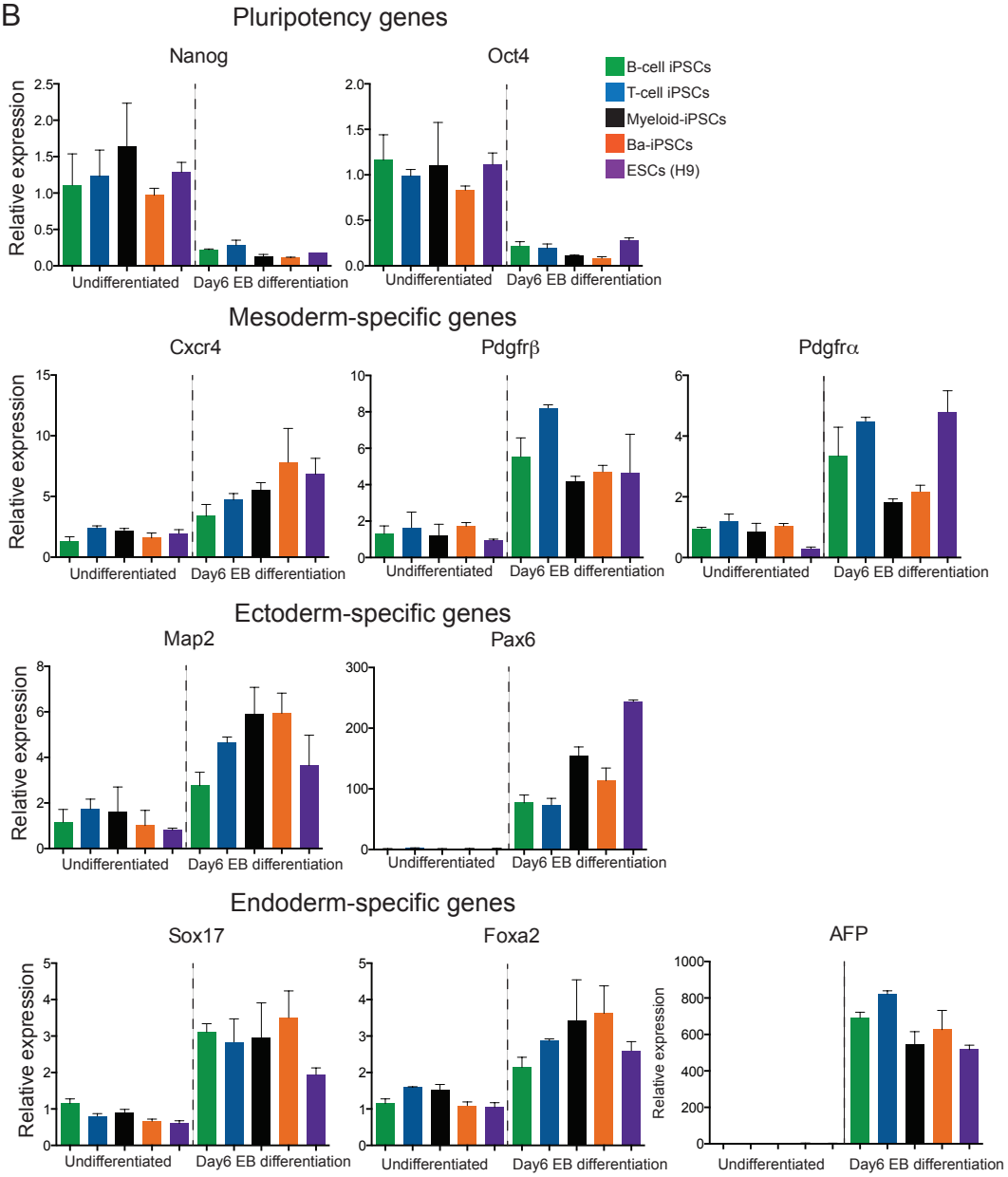
**g**


α-sm actin
FoxA2
β-III tubulin

A


iPS cells
Embryoid Bodies
day6
In vitro
differentiation
RNA extraction and
RT-qPCR

B


Pluripotency genes
Nanog
Oct4
B-cell iPSCs
T-cell iPSCs
Myeloid-iPSCs
Ba-iPSCs
ESCs (H9)
Relative expression
Undifferentiated
Day6 EB differentiation
Mesoderm-specific genes
Cxcr4
Pdgfrβ
Pdgfrα
Ectoderm-specific genes
Map2
Pax6
Endoderm-specific genes
Sox17
Foxa2
AFP