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Reprogramming of Human Peripheral Blood Cells to Induced Pluripotent Stem Cells

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Embryonic stem cells are pluripotent cells derived from the inner cell mass of the developing embryo that have the capacity to differentiate into every cell type of the adult (Evans and Kaufman, 1981; Martin, 1981; Martin and Evans, 1975; Thomson et al., 1998). The generation of patient-specific pluripotent cells is therefore an important goal of regenerative medicine. A major step to achieve this was the recent discovery that ectopic expression of defined transcription factors induces pluripotency in somatic cells (Lowry et al., 2008; Park et al., 2008b; Takahashi et al., 2007; Yu et al., 2007). Until now, the most common source from which to derive human iPSCs has been skin fibroblasts (Lowry et al., 2008; Park et al., 2008a, 2008b; Takahashi et al., 2007; Yu et al., 2009). However, the requirement for skin biopsies and the need to expand fibroblast cells for several passages in vitro represent a hurdle that must be overcome to make iPSC technology broadly applicable. Peripheral blood can be utilized as an easily accessible source of patient tissue for reprogramming. Here we derived iPSCs from frozen human peripheral blood samples. Some of the iPSCs had rearrangements of the T cell receptor (TCR), indicating that T cells can be reprogrammed to pluripotency.

Recently, granulocyte colony stimulating factor (G-CSF)-mobilized CD34⁺ blood cells have been used as a source from which to derive iPSCs (Loh et al., 2009). However, this requires the subcutaneous injection of G-CSF, a process that can be applied only if the donor is in good medical condition. Also, the negative effects of treatment of patients with growth factors such as erythropoietin (Miller et al., 2009) and G-CSF are still being investigated. Of concern is the use of G-CSF because this cytokine is

a growth factor for myeloid cell precursors (Touw and van de Geijn, 2007) and because G-CSF treatment of patients with severe congenital neutropenia (SCN) can result in a truncated G-CSF receptor allele and acute myeloid leukemia transformation (Touw, 1997). Derivation of iPSCs from peripheral mononuclear blood cells would circumvent all these issues; in addition, peripheral blood is the most accessible adult tissue and permits access to numerous frozen samples already stored at blood banks. Such samples could be expanded in culture and reprogrammed to iPSCs, which in turn allows researchers to study the molecular mechanism underlying blood and other disorders. We show here the derivation of iPSC clones from mature peripheral blood T and myeloid cells.

Mononuclear (MNC) blood cells were isolated from several donors by Ficoll-Hypaque density gradient centrifugation (Ferrante and Thong, 1980; Vissers et al., 1988). Samples were frozen and thawed days to several months after freezing and expanded in IL-7 or in G-CSF, GM-CSF, IL-6, and IL-3 for 5 days. In our initial experiments we used the FUW-M2rtTA and the individual doxycycline-inducible lentiviruses encoding Oct4, Sox2, c-Myc, or Klf4 (Brambrink et al., 2008). However, in ten independent experiments we were not able to reprogram peripheral blood cells with this system. One possibility for failure to obtain iPSCs is that peripheral blood cells are difficult to infect, reducing the probability of obtaining cells carrying the four factors as well as the FUW-M2rtTA construct. Also, we find that the efficiency of blood reprogramming (0.001%–0.0002%) is approximately 10–50 times lower than that of human fibroblast reprogramming.

To increase the infection efficiency, we used a doxycycline-inducible lentivirus encoding all four factors Oct4, Klf4, Sox2, and c-Myc from a polycistronic expression cassette (pHAGE2-TetOminiCMV-hSTEMCCA) (Sommer et al., 2010). Blood cells were simultaneously infected with a constitutively active lentivirus encoding the reverse tetracycline transactivator (FUW-M2rtTA) (Hockemeyer et al., 2008) as well as the polycistronic vector. Infected blood cells were transferred onto feeder layers of mouse embryonic fibroblasts (MEFs) and cultured in the presence of IL-7 or G-CSF, GM-CSF, IL-6, and IL-3 and 2 μ g/ml doxycycline (Dox) for an additional 4 days (Figure 1A). At day 5 after Dox induction, the cells were transferred to human ESC medium containing 2 μ g/ml Dox, and 25–40 days later colonies were picked and expanded.

We obtained iPSC colonies from several donors of different age (25–65 years) (Table S1 available online). We found that cells cultured in the presence of IL-7 expanded and reprogrammed more efficiently than cells grown in the presence of G-CSF, GM-CSF, IL-3, and IL-6. Southern blot analyses probing for M2rtTA vector integrations indicated that several iPSC lines of donors 3 (Figure S1A) and 4 (and data not shown) were derived from independent cells. Colonies were expanded into stable, Dox-independent iPSC lines that were not dependent on exogenous factor expression (Figure S1B). iPSCs that displayed the morphology characteristic of human ESCs had a normal karyotype (Figure S1C) and stained positive for the pluripotency markers Oct4, Nanog, and Tra1-81 (Figure 1B). Blood-derived iPSCs were cultured up to 35 passages and had elongated telomeres as shown by

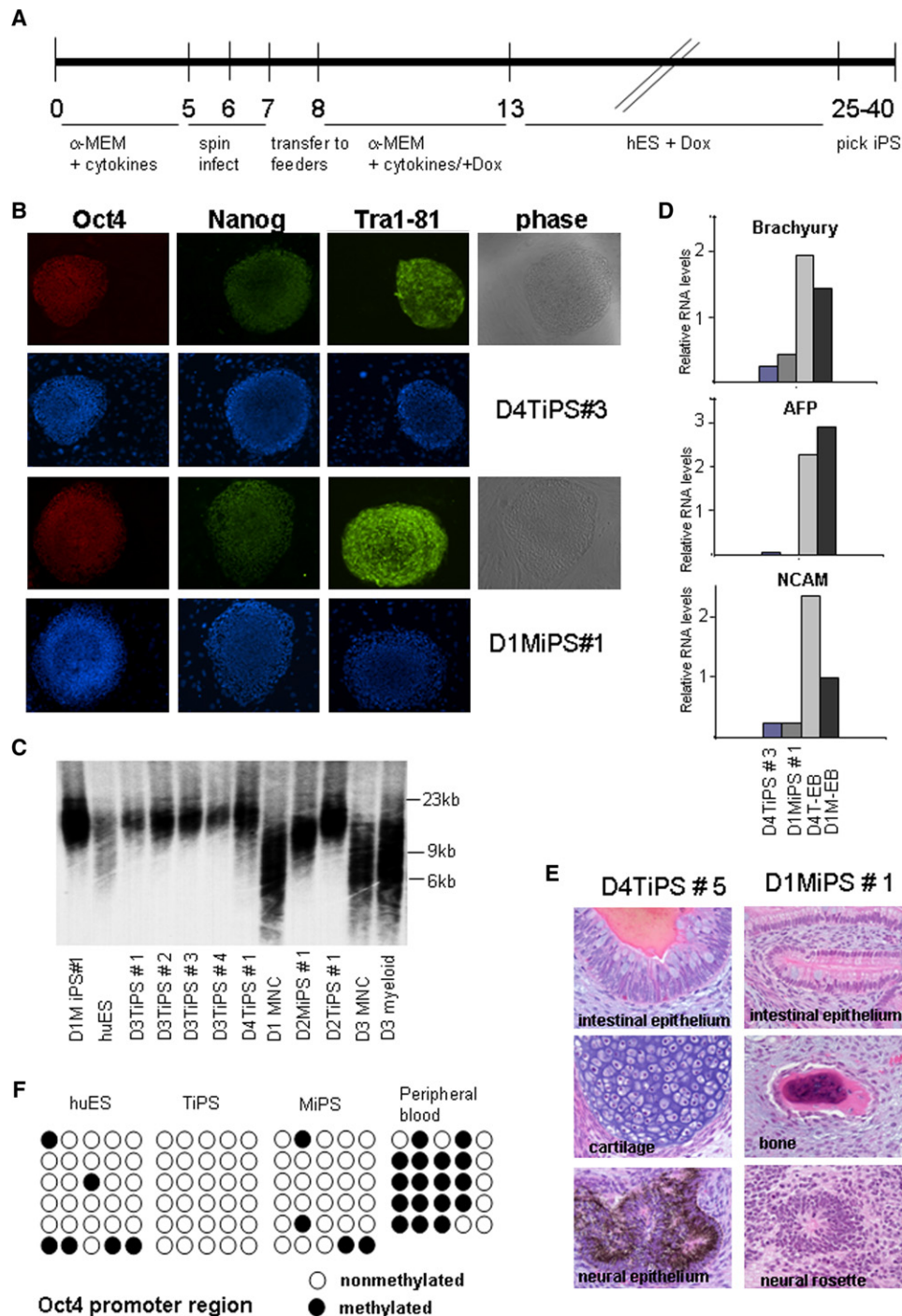


Figure 1. Characterization of Peripheral Blood-Derived iPSCs

(A) Schematic drawing demonstrating the strategy to derive iPSCs from human peripheral blood.

(B) Immunostaining for pluripotency markers Oct4, Nanog, and Tra1-81 in one representative T-iPSCs and M-iPSC line.

(C) Southern blot analyses showing that telomeres are elongated in T-iPSCs and MiPSCs compared to mononuclear (D1MNC, D3MNC) and myeloid (D3myeloid) cells.

(D) Quantitative RT-PCR assay for expression analyses of mesodermal (Brachyury), endodermal (AFP), and ectodermal (NCAM) lineage markers in differentiated embryoid bodies and iPSCs. Data are relative to GAPDH.

(E) Teratoma analysis from one representative T-iPSC and M-iPSC line.

(F) Methylation analyses of the Oct4 promoter region in human ESC, T-iPSC, M-iPSC, and peripheral blood cells.

See also Table S1 and Figure S1.

Southern blot analyses via an 800 bp TTAGGG repeat probe (Figure 1C; de Lange, 1992).

To assess the *in vitro* differentiation capacity of the iPSC lines, the cells were differentiated into embryoid bodies (EBs). Quantitative RT-PCR analyses of mesodermal (Brachyury), endodermal (AFP), and ectodermal (NCAM) markers demonstrated that all three lineage markers were upregulated in the differentiated EBs as compared to the undifferentiated iPSC lines (Figure 1D). To evaluate their *in vivo* differentiation potential, the iPSCs were injected subcutaneously into NOD-SCID mice and tumors were removed after 6–8 weeks. Histological analyses revealed that cell types characteristic for all three germ layers including ectoderm (neural rosette, neural epithelium), mesoderm (cartilage, bone), and endoderm (intestinal epithelium) were present (Figure 1E), indicative of pluripotency.

Reactivation of Oct4 locus through demethylation of its promoter during reprogramming is a hallmark of iPSCs. We therefore determined the methylation patterns of the Oct4 promoter region in iPSCs and peripheral blood cells. iPSCs had mostly nonmethylated promoter regions characteristic for the active Oct4 gene, whereas peripheral blood samples showed the expected highly methylated promoter (Figure 1F). The results shown in Figure 1 indicate that peripheral blood-derived iPSCs are pluripotent and show the molecular and morphological characteristics of human ESCs.

T cell development involves sequential genetic DNA rearrangements of the T cell receptor (TCRD > TCRG > TCRB > TCRA) or immunoglobulin loci (IGH > IGL > IGK), respectively (Davis and Bjorkman, 1988; Kiselow and von Boehmer, 1995; Rajewsky, 1996; Tonegawa, 1983). This allowed us to retrospectively assess whether the iPSCs were derived from mature T cells (TCR gene rearrangements), B cells (IG gene rearrangements), or myeloid cells (no TCR/IG gene rearrangements). During normal development, T cells mature in the thymus and migrate into the periphery as fully differentiated cells (Kiselow and von Boehmer, 1995). Detection of any TCR gene rearrangement in iPSCs derived from peripheral blood of healthy donors is, therefore, indicative of a mature T cell. PCR analyses were performed to detect potential

TCR delta (TCRD), TCR gamma (TCRG), or TCR beta (TCRB) rearrangements via TCR primer sets designed by the BIOMED-2 consortium (van Dongen et al., 2003) and purchased from *InVivoScribe Technologies*. The primer mixes target conserved regions within the variable (V), diversity (D), and joining regions (J) of the TCRB, TCRD, or the V and J regions of TCRG, respectively. In a clonal cell population, amplification of this region results in a PCR product within a predictable size range.

We identified bands within the valid size range for TCRB (Figure 2A; Figure S1D) and/or TCRG (Figure 2B; Figure S1E) gene rearrangements for all iPSC clones derived from donors 3 and 4. All clones analyzed tested negative for TCRD rearrangements (Figure 2C and data not shown). Sequencing analyses further identified the specific nature of productive TCRB and productive or unproductive TCRG gene rearrangements as shown in Figure 2D. Some PCR conditions led to amplification of additional bands inside and outside the valid size range. We cloned numerous PCR products that were in close proximity to the expected size range and confirmed that they reflect unspecific amplicons (data not shown).

One iPSC line (D1MiPS #1) derived in IL-7 was negative in all TCR gene rearrangement assays (Figures S2A–S2C). We therefore investigated whether this clone originated from a B lymphocyte or a myeloid cell. With the framework 1-3 primer sets (van Dongen et al., 2003), we did not detect any IGH gene rearrangements (Figure S2D), suggesting that this clone may have originated from a myeloid cell. As expected, the two iPSC clones (D2MiPS #1 and #2) derived in the presence of G-CSF, GM-CSF, IL-3, and IL-6 tested negative in all TCR (Figures S2A–S2C) or IGH gene (Figure S2D) rearrangement assays, whereas one iPSC line (D2TiPS#1) derived from the same donor in IL-7 tested positive for TCRG gene rearrangement (Figure S2C). In summary, our results indicate that iPSCs can be derived from terminally differentiated adult peripheral T cells.

Current protocols for reprogramming human cells are based on skin fibroblasts, keratinocytes, or G-CSF-mobilized CD34⁺ cells (Loh et al., 2009; Park et al., 2008b; Takahashi et al., 2007; Yu et al.,

2009). Nuclear transfer and four factor-mediated reprogramming experiments have demonstrated that pluripotency can be induced in terminally differentiated mouse lymphocytes (Hanna et al., 2008; Hochedlinger and Jaenisch, 2002; Hong et al., 2009). Here, we show that human peripheral blood T and myeloid cells cultured in IL-7 or G-CSF, GM-CSF, IL-3, and IL-6 can be reprogrammed to a pluripotent state via a polycistronic vector encoding Oct4, Klf4, Sox2, and c-Myc. Because of sequential DNA rearrangements of TCR or IG genes during lymphocyte development (Kiselow and von Boehmer, 1995; Rajewsky, 1996), we were able to retrospectively assess that the majority of iPSCs were derived from peripheral blood T cells. Two clones obtained in G-CSF, GM-CSF, IL-3, and IL-6 and one clone derived in IL-7 tested negative for TCR and IG gene rearrangements, suggesting that these iPSCs originated from myeloid cells. Proliferation of somatic cells is an important parameter of reprogramming (Hanna et al., 2009), which is consistent with the higher reprogramming efficiency of T lymphocytes as compared to myeloid cells because T cells have higher proliferation rates and better long-term growth potential *in vitro* than myeloid cells.

Our study demonstrates that peripheral blood can be utilized as an easily accessible source of patient tissue for reprogramming without the need to extensively maintain cell cultures prior to reprogramming experiments. This is an important step to make the iPSC technology more broadly applicable. Importantly, reprogramming of peripheral blood samples will permit access to numerous frozen samples already stored at blood banks. These samples are often of restricted use for research, because limited cell numbers do not allow experimental manipulations. This is particularly relevant if the patient is deceased and new material cannot be obtained. Generation of iPSCs from such samples could provide cell numbers large enough to retrospectively screen for genetic factors and to study molecular mechanisms underlying myeloid and lymphoid blood disorders. The culture conditions used in the current study primarily expanded T lymphocytes and myeloid cells and are probably the reason why B lymphocyte iPSCs were not derived. To expand B

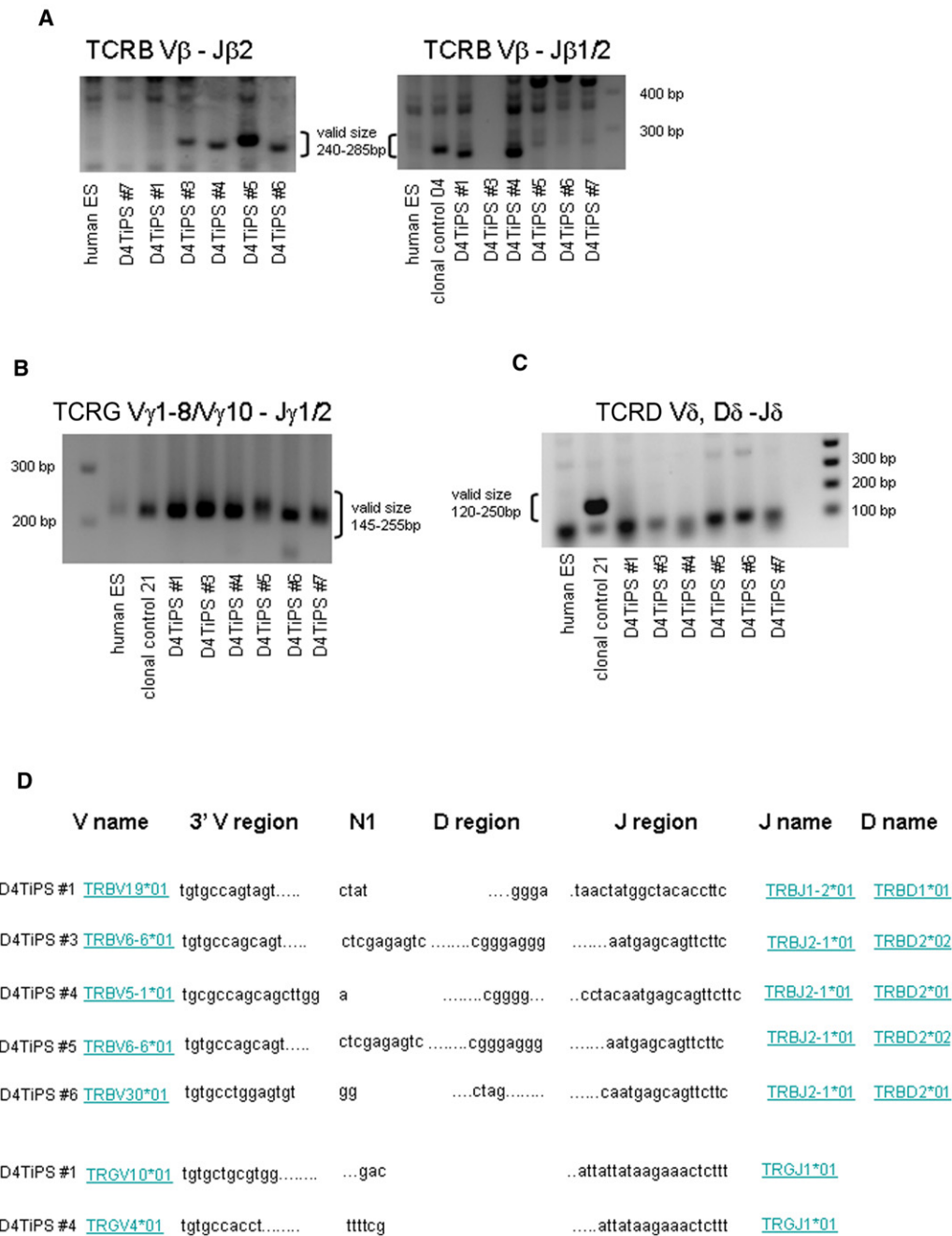


Figure 2. Analyses of TCR Gene Rearrangements in T-iPSCs

(A) PCR analyses of TCRB V β -J β gene rearrangements in six iPSC lines derived from peripheral blood of donor 4. PCR products within the indicated valid size range were cloned and sequenced.

(B) PCR analyses of TCRG V γ -J γ gene rearrangements in six iPSC lines derived from donor 4. PCR products within the indicated valid size range were cloned and sequenced.

(C) PCR analyses showing that all iPSC clones from donor 4 tested negative for V δ -J δ TCRD gene rearrangements.

(D) Sequencing analyses of PCR products obtained in (A) and (B). Shown are productive TCRB and productive (D4 #4) and unproductive (D4 #1) TCRG gene rearrangements in selected T-iPSC clones of donor 4. Genomic DNA from human ESC line BGO2 was used as negative control in all PCR assays; clonal positive controls were purchased from *InVivoScribe* Technologies.

See also Table S1 and Figures S1 and S2.

lymphocytes more efficiently, prior to the reprogramming process cells would need to be cultured in IL-4/CD40 ligand

in order to promote B cell survival and expansion (von Bergwelt-Baildon et al., 2002). In summary, our study allows re-

programming of the most easily available adult lineage and provides a protocol to access samples stored at blood banks.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, two figures, and one table and can be found with this article online at doi:10.1016/j.stem.2010.06.002.

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