



Abstract

Induced Pluripotent Stem (iPS) cell technology enables the creation of customized in vitro disease models, personalized genomic health analyses, and eventually, autologous cell therapy. Industry must be able to create thousands of iPS cell lines from tractable sources amenable to highly parallel processing in order to meet the anticipated demands of this technology for analytic and research applications. Human blood is one of the most common tissue sources because it is already found in approximately 3 ml aliquots at repositories worldwide. Therefore, it is one of the most tractable sources for reprogramming due to its accessibility.

We launched an effort to consistently create iPS cells from material that can be recovered from a 3 ml sample derived from the peripheral blood of multiple human donors. Here we present evidence for the creation of iPS cells from one of these donors. To this end we isolated T cells from non-mobilized, peripheral blood of a normal and healthy donor. These cells were then activated, expanded, and transduced with a cocktail of Moloney Murine Leukemia-derived retroviral (MLLV) particles independently encoding the defined reprogramming factors: Oct4, Sox2, c-Myc, and Klf4. Transduced cells were maintained in suspension in the presence of mouse embryonic fibroblasts (MEFs), and iPS-like colonies were observed after 3 weeks in culture. Six of the picked and clonally expanded colonies exhibited standard ES-like morphology, cell surface markers, AP staining and differentiated into a variety of cell types similar in efficiency to iPS cells derived from other cell sources such as fibroblasts (Fib-iPS). The iPS clones derived from T cells, referred to as "TiPS", maintain a normal karyotype as well as a genetic background that mimics the original donor material. Teratoma experiments are in progress to verify the ability of these cells to form tissue derivatives from all three primary germ layers.

The ability to generate iPS cells from standard whole blood samples that reflect the genetic identity of their hosts will pave the way for large-scale processing. The industrialization of this process will facilitate advances in research by providing the large number of cell lines necessary to satisfy the demands of the academic, clinical, and pharmaceutical community.

Reprogramming Process

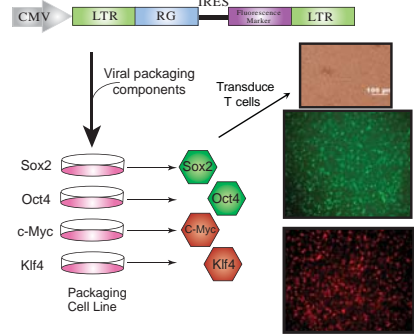


Figure 1
T cell Activation and Expansion—Peripheral Blood Mononuclear Cells (PBMCs). A leukocyte pack was processed via density gradient to yield PBMCs which were then expanded 3 days in serum free media + rhIL2 and anti-CD3 antibody. Exponential growth was verified and FACs analysis used to confirm that the bulk of the population bears T cell surface markers (see Figure 3). Cells were then transduced with a cocktail of VSVg pseudotyped retroviral particles expressing the defined factors for reprogramming.

Retroviral Vector—Each reprogramming gene is linked to a fluorescent marker by an IRES whereby the promoter within the Long Terminal Repeat (LTR) region drives the expression of a single transcript encoding a reprogramming gene (RG). Fluorescence is used to determine infection efficiency.

Retroviral Transductions—T cells were transduced with viral cocktail, IL2, and polybrene via centrifugation. Cells were incubated for 4 hours at 37 degrees and then given a partial media exchange and transduction was repeated the next day.

Plating Transduced T cells on MEFs—Efficiencies of transduction were assessed 3 days post transduction by determining the percent of total cells positive for fluorescence by flow cytometry. Transduced cells were transferred to plates containing mouse embryonic fibroblasts (MEFs) and supplemented with reprogramming media.

Maintenance and Feeding of MEF-plated Transduced Cells—Half-media exchanges were performed for each reprogramming plate with HES Media or MEF-conditioned media supplemented with zebrafish FGF. A novel feeding strategy was designed to minimize suspension cell loss while maximizing the positive effects of replenishing of media.

iPS Colony Identification—Well defined iPS colonies began to appear approximately 3 weeks post transduction, silencing of fluorescence within colonies was verified, and colonies were picked and transferred to fresh plates of MEFs.

Morphology of T cell-Derived iPS Cells (TiPS)

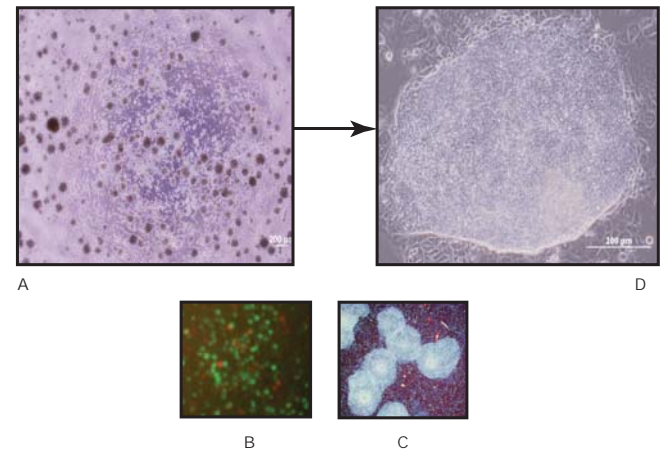


Figure 2
(A) Activated and expanding T cells display characteristic cell morphology and clustering behavior. (B, C, D) Detection of retroviral transduction efficiency is determined by GFP and RFP expression 72h post initial transduction, over the course of ~3 weeks the transgenes are silenced and display an hES cell phenotype.

TiPS Colonies Derived From Non-mobilized Peripheral Blood T cells

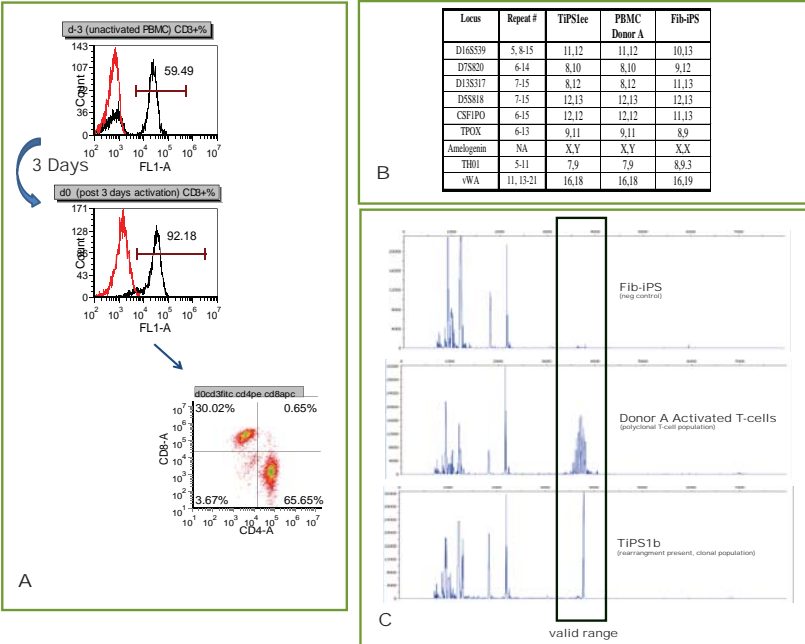


Figure 3
(A) Peripheral blood mononuclear cells (PBMC) from a non-mobilized donor are activated with anti-CD3 antibody and expanded in the presence of IL-2. The population is skewed predominantly towards T cells and then transduced with the reprogramming factors. (B) STR analysis shows that the iPS colonies are derived from the donor's genetic material. The donor PBMC and the iPS line are male gender specific and are identical to each other for 15 allelic polymorphisms across the 8 STR loci analyzed. (C) T cell receptor beta chain rearrangement analysis confirms the iPS colonies arose from T cells. T cells have a single productive V-J rearrangement in the TCR beta chain and should retain this characteristic gene sequence after becoming TiPS cells; using a master mix combining various primers for the most common beta chain rearrangements PCR amplification shows one band of unique size and sequence as determined by fragment analysis electropherogram on an ABI 3730 DNA analyzer. (iPS cells derived from fibroblasts, Fib-iPS)

Characterization of TiPS

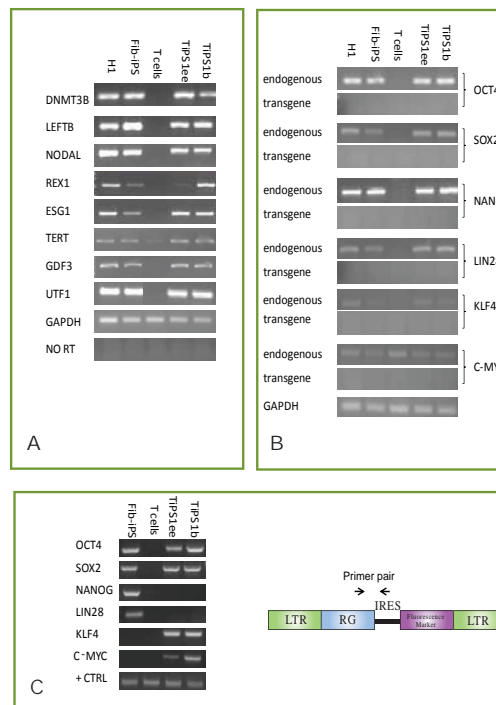


Figure 4
(A) TiPS clones express human embryonic stem cell marker genes. Total RNA was isolated from H1 hES cells, Fib-iPS (derived from fibroblasts), T-cells from the primary donor, and TiPS clones TiPS1ee and TiPS1b and analyzed using RT-PCR. (B) Endogenous and exogenous (transgene) expression of reprogramming genes show complete reprogramming as evidenced by silencing of transgene expression. GAPDH was used as amplification control in both A+B. (C) Genomic DNA was isolated and analyzed by PCR to confirm integration of reprogramming genes by using forward primers for the gene of interest and reverse primers for the IRES. OCT4 forward and reverse primers were used as a PCR reaction control.

Characterization of TiPS

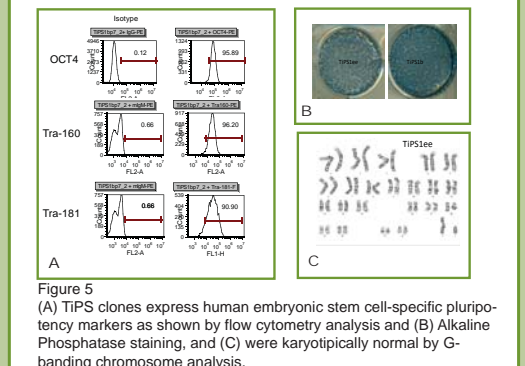


Figure 5
(A) TiPS clones express human embryonic stem cell-specific pluripotency markers as shown by flow cytometry analysis and (B) Alkaline Phosphatase staining, and (C) were karyotypically normal by G-banding chromosome analysis.

TiPS Differentiate Into Multiple Cell Types

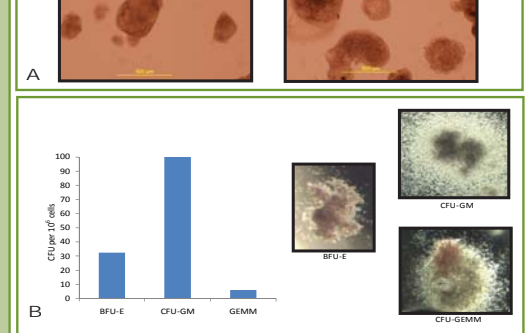


Figure 6
Cardiomyocytes—(A) TiPS clones form embryoid bodies (EBs) and can be differentiated into cardiomyocytes via HGF/bFGF mediated cardiac induction. Beating aggregates were observed on day 14. Blood—(B) Hematopoietic progenitor cells (HPCs) can also be derived from EBs using a combination of BMP-4, VEGF, Flt-3 ligand, IL-3, GM-CSF and FGF-2. Functional capability of TiPS1ee-derived HPCs was determined using the colony-forming unit (CFU) assay. CFU-GM, BFU-E, and CFU-GEMM colonies were observed at day 12.

Sources of Cells



Thousands of biorepositories throughout the world have been established by private and public institutions, and the utilization of such facilities for research in genomics and proteomics is well established. Biorepositories typically house human peripheral blood, bone marrow, and/or tissue biopsies. In the U.S., the National Cancer Institute's (NCI) Office of Biorepositories and Biospecimen Research (OBBR) has begun to "coordinate efforts to improve the availability and quality of human specimens needed for research supported by the NCI throughout all its programs." <http://biospecimens.cancer.gov/about/default.asp>

The advent of iPS technology and the work presented here raises the possibility that cryopreserved specimens might serve a wider range of research including cellomics. It has been shown that viable PBMCs have been recovered from biorepositories following cryopreservation for up to 12 years*.

*Viability and recovery of peripheral blood mononuclear cells cryopreserved for up to 12 years in a multicenter study by: Kleiberger C.A. et al. Clinical and Diagnostic Laboratory Immunology, Vol. 6, No. 1. (1999)

The Promise of iPS Cell Technology

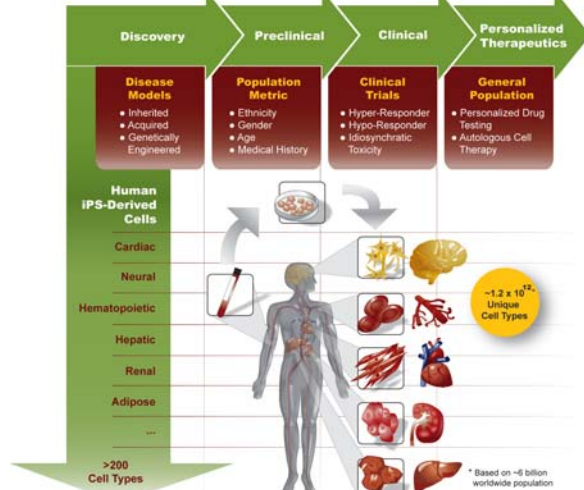


Figure 7
The left axis and the central figure represent the different cell types within the human body while the top axis represents areas of drug discovery and clinical use.

Summary

- Successfully generated iPS cells from T cells derived from the peripheral blood of a non-mobilized donor
- Amount of starting material is adaptable to 1 ml of starting material from a standard vacutainer
- TiPS reflect identity of host material
- TiPS harbor hallmark characteristics of normal human ES cells and iPS derived from other cell sources
- TiPS are competent for differentiation into multiple cell types

Conclusion

The advent of reprogramming technology and the work presented here raises the possibility that pre-existing cryopreserved specimens could be harnessed for reprogramming to accelerate research and meet the demands of industrial, pharmaceutical, and clinical communities. Future studies at CDI will seek to demonstrate the utility of cryopreserved PBMC's for reprogramming studies.