Reprogramming T cell Lymphocytes to Induced Pluripotent Stem Cells

A THESIS
SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

Jakub Tolar

December 2012

Acknowledgements

I deeply thank my advisor, Dr. Jakub Tolar, for being an excellent mentor and giving me the valuable opportunity to be part of his laboratory. I would like to thank Cindy Eide for her patience and guidance during the past year and Christopher Lees for his assistance during my projects. Also, I would like to thank Dr. Jonathan Slack and Susan Keirstead for their guidance and for giving me the opportunity to be part of the Stem Cell Biology program and fulfill my goals. Finally, I would like to thank my family and friends for their unconditional love and support during this journey.

Abstract

The discovery of induced pluripotent stem cells (iPSC) provided a novel technology for the study of development and pharmacology and complement embryonic stem cells (ES) for cell therapy applications. Though iPSC are derived from adult tissue they are comparable to ES cells in their behavior; multi-lineage differentiation and self-renewal. This makes iPSC research appealing because they can be studied in great detail and expanded in culture broadly. Fibroblasts were the first cell type reprogrammed to an iPSC using a retrovirus vector, since then alternative cell types including lymphocytes have been used to generate iPSC. Different types of vectors have also been developed to enhance iPSC formation and quality. However, specific T lymphocyte subsets have not been shown to reprogram to a pluripotent state to date. Here, we proposed to derive iPSC from peripheral blood effector and central memory T cells, reasoning that the resultant iPSC will maintain the epigenetic memory of a T lymphocyte, including the T cell receptor (TCR) gene rearrangement. This epigenetic memory will enable the differentiation and expansion of T cell iPSC into professional T cells containing a specific TCR. These could then be used for cell therapy to target specific antigens, as well as to improve culture techniques to expand T cells *in vitro*. We studied different gene delivery methods to derive iPSC from different types of T lymphocytes. We assessed the viability of viral transduction using flow cytometry to detect green fluorescent marker contained in the viral construct and quantitative real time polymerase chain reaction (qRT-PCR) to detect Oct4, Klf4, Sox2, and c-Myc gene expression. Our results demonstrate that the Sendai virus construct is the most feasible platform to reprogram T lymphocytes. We anticipate that this platform will provide an efficient and safe approach to derive iPSC from different T cell subsets, including memory T cells.

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List of abbreviations

AP – Alkaline phosphatase

bFGF – Basic fibroblast growth factor

BMT – Bone marrow transplant

cDNA – Complementary deoxyribonucleic acid

DAPI – 4',6-diamidino-2-phenylindole

DMEM – Dulbecco's modified eagle medium

DMSO – Dimethyl sulfoxide

DNA – Deoxyribonucleic acid

Dox – Doxycycline

ESC – Embryonic stem cells

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FB – Fibroblasts

FBS – Fetal bovine serum

GFP – Green fluorescent protein

GVHD – Graft versus host disease

HEPES – 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

hES – Human embryonic stem cell

HSC – Hematopoietic stem cells

IL-2 – Interleukin 2

iMEF – irradiated mouse embryonic stem cells

iPSC – Induced pluripotent stem cells

IRES – Internal ribosome entry site

LB – Luria-Bertani media

LV – Lentivirus

MEF – mouse embryonic stem cells

MHC – Major histocompatibility complex

MOI – Multiplicity of infection

NK – Natural killer cells

OSKM – Oct4, Sox2, Klf4, cMyc

PBMC – Peripheral blood mononuclear cells

PBS – Phosphate buffer saline

qRT-PCR – Quantitative real time polymerase chain reaction

RNA – Ribonucleic acid

RV – Retrovirus

T reg – Regulatory T cells

T_{CM} – Central memory T cells

TCR – T cell receptor

T_{EM} – Effector memory T cells

TM-LCL – EBV-transformed lymphoid cell line

UCB – Umbilical cord blood

VSV-G – Vesicular stomatitis virus - glycoprotein

INTRODUCTION

There are three landmarks in the field of developmental and stem cell biology: somatic nuclear transfer, embryonic stem cells and induced pluripotent stem cells. They contributed to one another and its because these finding that today we are able to use stem cells in regenerative medicine as a tool for treating life threatening diseases.

1. Somatic nuclear transfer, transcription factors and lineage switching

Somatic nuclear transfer was introduced by John Gurdon, when his laboratory generated tadpoles by nuclear transfer of intestinal cells nucleus to unfertilized eggs (Gurdon, 1958). The same concept was proven in mammals, when Wilmut and colleagues reported the cloned mammal generated by somatic nuclear transfer of epithelial cells to eggs (Wilmut *et al.,* 1997). These experiments showed that the genome of a differentiated state still has the genetic information required to derive an entire organism (Yamanaka, 2012). But what factors does the egg cytoplasm contained to be able to reprogram somatic cell nuclei? This is where the field of developmental and stem cell biology expanded to the study of cell fate and induced lineage conversion experiments. Metaplasia is a term used to describe any tissue-type-switching (Slack, 2007). One proposed theory for metaplasia is that the tissue type will change if the code is changed, whether by mutation, epigenetic switch or environmental effects (Slack, 2009). The proposed theory could explain the key factors that contribute to cellular reprogramming. Studies with *Drosophila* showed that ectopically expressed transcription factors were able to substitute one part of the body to another, changing cell fate (Scheneuwly *et al.,* 1987). At the same time this concept was shown in humans with the transcription factor MyoD, which converted fibroblasts into myocytes (Davis *et al.,* 1987). These experiments are examples of metaplasia and show that cell fate can be switched or even revoked to go back to a pluripotent state by changing the code with key transcription factors, also called “master regulators”.

2. Embryonic stem cells

Embryonic stem cells (ESC) are cells derived from the inner cell mass of the blastocyst stage of the embryo cultured *in vitro*. ESC were first derived from mouse (Evans and Kaufman, 1981) and later from humans in 1998 by Thomson and his group (Thomson *et al.,* 1998). Both human and mouse ESC led to the discovery by other groups that culture conditions with leukemia inhibitory factors and basic fibroblast growth factor enabled the maintenance of pluripotency of ESC *in vitro* (Yamanaka, 2012) showing that environmental factors also contribute to cell fate. ESC are pluripotent in nature, which permits them to differentiate to all primitive embryonic germ layers and they can self-renew (Stojkovic *et al*., 2004). These two characteristics make ESC to be of great interest, holding promise for the study of development, regenerative medicine, and disease modeling. Regenerative medicine is one of the major interests of stem cell research, using pluripotent cells for regeneration and tissue replacement in injury or disease. However, the use of human ESC have different ethical and legal perspectives do to the source of these cells; human embryos. Recently ESC-like pluripotent stem cells have been derived from adult tissues (e.g. induced pluripotent stem cells and tissue specific stem cells) holding similar promises.

3. Induced pluripotent stem cells (iPSC)

The discovery that an egg cytoplasm was able to reprogram a somatic cell nucleus when injected into an egg (nuclear transfer) (Gurdon, 1958) giving rise to a whole organism expanded the stem cell field to the study of induced lineage conversion leading to the later success of iPSC technology in mouse (Takahashi and Yamanaka, 2006). iPSC are derived from normal somatic cells that have been manipulated by the introduction of genes that encode pluripotent behavior, defined by Takahashi and Yamanaka as Oct4, Sox2, Klf4 and cMyc (OSKM). iPSC resemble ESC in the expression of specific pluripotency genes, surface markers, morphology and *in vivo* behavior. iPSC technology was appealing for human studies and a year later Takahashi and Yamanaka, simultaneously with Thomson's group, reported the generation of iPSC from adult human

fibroblasts using the same four genes (Takahashi *et al*., 2007). These finding opened new opportunities in disease modeling, epigenetic and developmental studies, drug screening and suggested a possibility of highly expanded personalized iPSC for autologous cell therapy, thus eliminating immune complications of allogenic cell transfer.

Human induced pluripotent stem cells derived from hematopoietic cells

Human iPSC were first derived from fibroblasts (Takahashi *et al*., 2007) and ever since a number of cell types have been reprogrammed to iPSC, including hematopoietic cells. Human blood progenitor cells (CD34+) were the first blood cells to be reprogrammed to iPSC (Loh *et al*., 2009). Blood draw is a common procedure in clinics and hospitals, thus it is accessible and it is a minimal invasive procedure compared to skin biopsy to harvest fibroblasts. However, progenitor cells are 0.03-0.09% of leukocytes making them a challenging cell source. Thus, mature blood cells are more feasible for these types of experiments. The first mature cell types from the hematopoietic lineage that were reprogrammed to iPSC were peripheral blood mononuclear cells (PBMC) (i.e., lymphocytes, monocytes and macrophages). Furthermore, immunoglobulin gene analysis showed that some of the iPSC were derived from T cells (Loh *et al*., 2010). In 2010, Brown *et al*. showed that human peripheral blood T lymphocytes can be reprogrammed to a pluripotent state with the use retroviral vector with OSKM factors. Because integrating vectors, including retroviral vectors, can mediate insertional mutagenesis scientists have developed alternative methods for reprogramming including non-integrating vectors (e.g., Sendai virus, plasmid vectors, miRNAs). In 2011, Seki *et al*. published the generation of iPSC from mature human peripheral blood T cells using a Sendai virus vector, thus, avoiding genomic transgene integration and suggesting a superior method for iPSC for pre-clinical applications. During the years it has been shown that cell maturity correlates with efficiency of iPSC formation. This was true for nuclear transfer experiments in mammals as well (Hochedlinger and Jaenisch, 2002). DNA methylation status, epigenetic modifications, chromatin condensation and telomere length account for the difficulty of reprogramming the nucleus of mature cells (Gurdon and Wilmut, 2011). Thus, the development of different reprogramming methods,

including culture conditions and vectors to enhance iPSC formation on differentiated cells is an ongoing area of research on the stem cell field.

Hematopoietic stem cells

Many tissues in our bodies have stem cells that maintain their integrity. These stem cells are called "tissue specific stem cells" and they are different from ESC. Tissue specific stem cells are multipotent, differentiating into a specific lineage (Slack, 2005). Hematopoietic stem cells (HSC) are one example; they reside in the bone marrow and give rise to all types of blood cells. Bone marrow transplantation (BMT) was the first stem cell therapy ever done in humans by E.D Thomas. BMT was used for treating blood related disease, like leukemia. E.D. Thomas and R.A. Good proved that bone marrow cells from a healthy donor were able to repopulate the bone marrow of a conditioned recipient. Thus, a donor graft contained enough HSC to establish fully functional hematopoietic system providing a proof of concept for stem cell based therapies.

T lymphocytes

Within the hematopoietic system, T cells play an important role in immune response to foreign antigens and each T cell subset (e.g. regulatory T cells, helper T cells (T_H), memory T cells) has a distinct function in the response. All of these subsets form the pool of peripheral blood T cells. In this manuscript we are going to focus on two T cell subsets, regulatory T cells and memory T cells.

1. Regulatory T cells

Regulatory T cells (T regs) suppress adaptive immune response and maintain immune homeostasis. T regs are immunosuppressive and increasing evidence supports their capacity to modulate immune response in BMT. Mice studies shows that T reg therapy inhibit acute or chronic graft versus host disease (GVHD) (Anderson *et al.,* 2004; Zhao *et al.,* 2008). In fact, human clinical trials of T reg therapy before or after BMT or cord blood transplantation have reported improvement of chronic GVHD, low incidence of acute GVHD, accelerated immune reconstitution and lower incidence of tumor relapse

(Trzonkowski *et al*., 2009; Di Ianni *et al*., 2011). Hence, T regs are of great interest in translational research. However, some of the obstacles for T reg therapy are their low abundance, limited expansion in short-term cultures, outgrowth of conventional T cells when cultured *in vitro* (Tang *et al*., 2011; Haque *et al*., 2012), and the fact that they have no unique cell surface markers (Riley *et al.,* 2009).

2. Memory T cells

Memory T cells defend the organism from foreign antigens by recognizing recurrence of disease. During immune response, naïve T cells differentiate into specific subsets (T_H1 and T_H2) performing their effector function. During the contraction phase of the immune response a small percentage of effector T cells become memory cells. Memory cells accumulate over the life span of an individual in so called memory pool and they can react and expand faster on a successive encounter with the foreign antigen. There are two classes of memory cells, effector and central and they are distinct in their homing and effector functions. Effector memory T cells (T_{EM}) migrate to inflamed peripheral tissues and perform effector functions. Central memory T cells (T_{CM}) migrate to secondary lymphoid organs where they proliferate and differentiate to effector cells. Even though these two types of memory cells are well defined in their surface markers and cytokine production, their development and maintenance is still unclear (Sallusto *et al*., 2003). Memory is the distinctive feature of the adaptive immune response. Thus, understanding the biology of memory cells is critical for successful immunotherapy.

3. T cell receptor repertoire

During T cell development the V, D, and J gene segments in human chromosomes 2, 14 and 22 rearrange to form the T cell receptor (TCR) which in the majority of T cells contains a β and α chain. First the D_β genes and the J_β genes rearrange followed by the rearrangement of V_β genes with DJ_β. The α chain rearrangement follows and multiple V_α gene segments rearrange with approximately 60 J_α gene segments producing a repertoire of TCR (Janeway, 2011). The VDJ rearrangement of T cells is helpful for the

study of lineage conversion and epigenetics. Moreover, it is a marker of epigenetic memory that is maintained in iPSC derived from T cells (Brown *et al.,* 2011; Loh *et al.,* 2010). During an immune response TCR recognize MHC class I or II molecules containing a non-self peptide in antigen presenting cells. Upon exposure to co-stimulatory molecules (e.g.,CD28) and cytokines (e.g., IL-2) T cells expand *in vitro* and *in vivo* and differentiate. Different expansion protocols have been proposed to stimulate and expand T cells and their subsets (Godfrey *et al.,* 2004; Hippen *et al.,* 2011) yielding high number of cells within days making this process amenable to research and clinical use.

Induced pluripotent stem cells from peripheral blood T cells

We propose to combine the T cell expansion with inducible pluripotency to enhance T reg and memory T cell expansion, study T cells on an epigenetic scale, to model preparation of autologous T cells for immunotherapy. In addition, Tcell-iPSC could be gene manipulated to contain a specific TCR of interest and differentiated in vitro into different T cell subsets with TCR specificity towards a given antigen. Many tumors (e.g. breast and prostate) have specific surface peptides that can be recognized by a TCR. If one can manipulate the VDJ genes to create a particular TCR of interest, it can have relevant therapeutic implications for cancer and cell therapy. Also, the possibility of gene editing a TCR would open new possibilities to enhance the expansion protocols for different T cell types enabling production of specific T cells that respond to a specific peptide in antigen presenting cells. Another reason for making iPSC from T cells is to delineate epigenetic changes during the reprogramming process. T lymphocytes are a large group of cells containing many subsets (e.g. T_H1, T_H2, T regs, memory cells) enabling the comparison of epigenetic changes from different subsets of the same lineage.

In this study, we evaluate different gene delivery methods including integrating and non-integrating vectors for reprogramming T cells from peripheral blood: whole T cells and T cell subsets such as regulatory and memory T cells. We hypothesized that T cell subsets,

as well as whole T cells, can be reprogrammed to iPSC delivering the four factors (Oct4, Sox2, Klf4, and cMyc) using integrating and non-integrating vectors. Our ultimate goal is to derive iPSC from central and effector memory T cells. Such iPSC are expected to maintain the epigenetic memory of the original T cell along with the specific TCR gene rearrangement. We show that Sendai virus is superior to retrovirus, lentivirus and minicircle vectors in the generation of T cell-iPSC. Experiments are ongoing to extend this observation to T regs, T_{CM} and T_{EM}.

METHODS

T lymphocyte expansion

Peripheral blood from a healthy human donor was collected by venipuncture using heparin as anticoagulant. Peripheral blood mononuclear cells (PBMCs) were isolated by density gradient centrifugation using Histopaque -1077 (Sigma, St. Louis, Missouri) and centrifuging at 1200 revolutions per minute (rpm) for 20 minutes on a 50 milliliters conical tube. Buffy coat was collected and cells counted on a Beckman Coulter Counter, Z1 (Beckman, Fullerton, California). A concentration of 0.5×10^6 of peripheral blood mononuclear cells were plated on 6 well plates coated with purified NA/LE mouse anti-human CD3 antibody (BD Pharmingen™, 555329, San Diego, California) and cultured with T cell medium: RPMI, 11.5% Fetal Bovine Serum (FBS), 1% L-glutamine, and 2% HEPES (filtered through a 0.22μM filter) for a minimum of three days at 37°C and 0.5% CO_2.

T regulatory cells expansion (Re-stimulation of frozen cells)

Peripheral blood regulatory T cells (T regs) were kindly provided by Dr. Kelli Hippen (UMN). T regs stored at -80°C were thawed and centrifuged at 1300 rpm for 5 minutes. Cell pellet was resuspended in X-vivo AB medium and a total of 2×10^6 cells were cultured in a T-75 flask with 4ml of media containing IL-2 cytokine at a concentration of 300 IU/mL. In addition, anti-CD3/anti-CD28 beads were added to the culture at a 1:3

(Tregs:beads) ratio. The following day 4 mL of media with IL-2 was added to the culture and incubated in 37°C. Medium was added to the culture every two days to maintain a concentration of 0.5×10^6 cells/mL.

Effector memory and Central memory T cell expansion

Effector memory (EM) and central memory (CM) expansion is a co-culture system that consists of irradiated Epstein Barr virus-transformed lymphoblastoid cell line (TM-LCL) cells, irradiated PBMCs and the EM or CM cells. (Dr. Michael Jensen Laboratory, Seattle, WA, kindly provided the memory T cells and the standard operating procedure).

1. Preparation of TM-LCL feeder cells

Frozen TM-LCL were thawed and transferred to a 50 mL conical tube with T cell medium. Cells were then centrifuged at 1200 rpm for 10 minutes and cell pellet resuspended in 2 mL of T cells medium. Cells were counted and 0.5×10^6 cells per milliliters were plated on a T-25 flask with T cell medium. Cells were monitored and split two times a week to maintain this concentration. When ready, 5×10^6 cells were irradiated at 8000 cGy and placed on the co-culture system (a T-25 flask with a final volume of 25 mL of T cell media).

2. Preparation of PBMC

PMNCs were isolated from whole blood of a healthy donor by density gradient centrifugation using Histopaque -1077 (Sigma, St. Louis, Missouri) and centrifuged at 1200 revolutions per minute (rpm) for 20 minutes on a 50 mL conical tube. Buffy coat was collected and cells counted on a Beckman Coulter Counter, Z1 (Beckman, Fullerton, California). A total of 25×10^6 cells were irradiated at 3500 cGy and placed on the co-culture system.

3. Preparation of EM and CM cells

EM and/or CM cells were thawed and transferred to a 50 mL conical tube containing T cell medium. Cells were centrifuged at 1200 rpm for 10 minutes and cell pellet resuspended on 1-2 mL of T cell medium for counting. A total of 1×10^6 EM or CM cells were added to the co-culture system. In addition, anti-CD3 was added to the system at a concentration of 30ng/mL. The co-culture system was incubated at 37°C with 5% CO_2 and the next day and every 48 hours thereafter 25U/mL of IL-2 was added to the culture. Cells were moved to a T-75 flask when the concentration reached 0.8 to 1.2×10^6 cells/mL with a final volume of 50 mL and this concentration is maintained by splitting the cells one or two times a week for 14 days.

Transforming competent cells

One Shot Stbl3 Chemically Competent *E.coli* (Invitrogen, Carlsbad, California) were transformed with the plasmid of choice following manufacturers protocol. Transformed bacteria were incubated in Luria-Bertani (LB) agar plates containing ampicillin at a concentration of 100 ug/mL overnight at 37°C.

Plasmid Expansion

Colonies were isolated and cultivated in 2.5 mL of LB medium with ampicillin [100 μg/mL] for 8 hours at 37°C on shaker. A bigger stock was made after incubation by transferring 100 μL from the small stock to 400 mL of LB medium with ampicillin and incubated overnight at 37°C on shaker. The next day DNA was isolated using PureLink HiPure Plasmid Filter Purification Kits (Invitrogen) following manufacturers protocol. The amount of DNA obtained was measured using Nano Drop Lite spectophotometer (Thermo Scientific, Waltham, MA).

Restriction Enzyme digest

For quality control of plasmid, a restriction enzyme digest was done. In a microfuge tube 1μL of 10x Buffer, 1μL of DNA, 7 μL of distilled water and 0.5μL of enzyme (BioLabs, New England) were mixed for a total of 10μL. The tubes were incubated for 1 hour at

37°C. The agarose gel was prepared during the incubation. A 200 mL flask was used to combine 50 mL of 1x TAE buffer (Tris-acetate + EDTA) and 0.5 g of agarose. The mixture was heated until homogeneous and 2.5μL of ethidium bromide was added to the solution. The gel was poured into the agarose plate. After 1-hour incubation 2μL of loading dye is added to each tube. The ladder tube was prepared by adding 7μL of water, 1μL of ladder DNA and 2μL of loading dye. The gel was loaded with 12μL of each sample and ran for 15 to 30 minutes at 115 volts. The gel was analyzed on the Alphalmager HP (Alpha Innotech, San Leandro, CA), a UV transilluminator.

Viral production

Viral production was done by transfecting 293T.17 cells with Lipofectamine 2000 (Invitrogen) with three plasmids: one containing the gene of interest (also called the "cargo plasmid"), a plasmid expressing the VSV-G envelope gene and a helper plasmid with the retroviral Gag/Pol gene. The day before lipofection, 293T.17 cells were plated in T150 flasks at a concentration of 20×10^6 cells per flask. The next day each flask received a media change and 10 mL of fresh DMEM 10% FBS was added to the flasks. In a 15 mL conical tube 1 mL of Opti-MEM medium (Invitrogen) was incubated with 48 μL of lipofectamine 2000 for 5 min. In another conical tube 1 mL of Opti-MEM is mixed with plasmid DNA in the following concentration: 1.50 μg of envelope, 6.75 μg of gag/pol and 7.50 μg of cargo plasmid. The two mixtures are mixed together and incubated for 20 min and transferred to each flask. Supernatant containing virus was harvested 48-72 hrs after lipofection and filtered through a 0.45 μM filter. 10 mL of DMEM 10% FBS was added to each flask and incubated for an additional 24 hrs for a second harvest. For Lentivirus, Lenti-X-Concentrator (Clontech) was used following manufacturers protocol to concentrate the virus. All viruses were either used fresh or frozen at -80°C.

Envelope plasmids used

1. VSV-G provided by George Q. Daley laboratory (Boston, Massachusetts)

Gag/Pol plasmids used

1. 8.91 provided by George Q. Daley laboratory. Used for Lentivirus production.
2. 8.2 provided by George Q. Daley laboratory. Used for Retrovirus production.

Cargo plasmids used (All p-mig plasmids: (Park *et al.,* 2008; Park *et al.,* 2009))

1. FUW-M2rtTA (Plasmid 20342) from Addgene
2. TetO-FUW-OSKM (Plasmid 20321) from Addgene
3. p-mig-Oct4 provided by George Q. Daley laboratory
4. p-mig-Sox2 provided by George Q. Daley laboratory
5. p-mig-Klf4 provided by George Q. Daley laboratory
6. MSCV h c-MYC IRES GFP (plasmid 18119) from Addgene

Viral titer

Retrovirus: A 6 well plate was coated with 293T cells at a concentration of 1×10^5 per well with 2 mL of DMEM 10% FBS medium and incubated overnight at 37°C, 5% CO_2. The next day fresh virus was added to each well at the following concentration: 0 μL, 2 μL, 20 μL, and 200 μL. Protamine sulfate was added to the wells at a concentration of 8 μg/mL. The plate was incubated at 37°C, 5% CO_2 for 24-72 hours. The cells were harvested using 0.05% trypsin/EDTA. Flow Cytometry using FACS Calibur was done to measure the amount of GFP positive cells.

Lentivirus: The presence of lentiviral p24 protein was detected using Lenti-X™ GoStix™ (Clontech, Mountain View, CA). The sensitivity of the test is 5×10^5 IFU/mL.

Mouse embryonic fibroblasts feeder cells (MEF)

Mouse embryonic fibroblasts (EmbryoMax PMEF P3 strain CF-1 Millipore, Billerica, MA) were thawed and plated on gelatin coated T-150 flask at a concentration of 1×10^6 cells per flask. The cells were cultured with 20 mL of DMEM 15% FBS and incubated at 37°C, 5% CO_2. The media was changed the day after and the flasks were incubated for another 72 hours were the cells were harvested using 0.05% trypsin-EDTA and split in a

1:3 ratio. The media was changed every 48-72 hours until the cells were 100% confluent. Cells were harvested on a 50 mL conical tube using 6 mL of 0.05% Trypsin-EDTA per flask and centrifuged at 1300 rpm for 5 minutes. The pellet was resuspended in 5 mL of DMEM 10% FBS and cells were irradiated with the following settings: 12.5 mA, 320 kV and 5500 cGy (XRad 320). After irradiation the cells were frozen at -80°C at a 1:1 ratio of freezing solution (80% FBS and 20% DMSO) and cells.

At the time of use, plates were coated in ultra pure water with 0.1% gelatin (Millipore, Billerica, MA) and incubated at room temperature for 30 minutes. A total of 1.5×10^6 MEFs were plated per 6 well plates with 2 mL of DMEM 10% FBS medium per well. The plates were incubated at 37°C, 5% CO_2 for up to 5 days.

Preparation of MEF conditioned media

Irradiated mouse embryonic fibroblasts (iMEFs) at a concentration of 4×10^6 cells were plated on a 0.5% gelatin coated T-75 flask with DMEM 10% FBS. The following day medium was replaced with 37.5 mL of hES media supplemented with 4 ng/mL of bFGF, and incubated in 37°C, 5% CO_2. The medium was replaced everyday for up to 7 days. The collected medium was stored unfiltered on 50ml conical tubes at -80°C. The media was filtered using a 0.22 μM filter at the time of usage and fresh bFGF (4 ng/mL or 100 ng/mL) was added considering depletion of this protein during long periods of storage. (Bresa Gen Inc. 2004, Human Embryonic Stem Cell Protocols).

Genomic expression analysis

1. Total RNA Purification (PureLink™ RNA Mini Kit, ambion by Life Technologies)

Cells at a concentration of 1×10^5 to 1×10^6 were transferred to an RNase-free tube and centrifuged at 2,000 x g for 5 minutes at 4°C. Lysis Buffer with 2-mercaptoethanol was added to the pellet at a volume of 0.3 mL and vortexed until cells appeared lysed. 70% ethanol was added and homogenized by vortexing. The sample was transferred to a spin cartridge and centrifuged at 12000 x g for 15 seconds. Wash buffer I was added to the spin cartridge and centrifuged at 12000 x g for 15 seconds. The spin cartridge was placed on a new collection tube and 500 μL of wash buffer II was added following a

centrifugation of 12000 x g for 15 seconds. The last step was repeated once again and 50 μL of RNase-free water was added to the center of the spin cartridge to elute the RNA that is bound to the membrane. After 1 minute incubation at room temperature, the tubes were spun for 2 minutes at 12000 x g to eliminate any genomic DNA contamination the collected eluates were DNAse treated with 50 μL of 10X turbo DNAse buffer containing 200 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, and 5 mM $CaCl_2$ and 1μL of turbo DNase enzyme (TURBO DNA-*free* Kit, Ambion). Samples were incubated for 30 minutes in a 37°C water bath. After incubation, 5 μL of DNase inactivation reagent was added to the sample and incubated at room temperature for an additional 2 minutes. Samples were centrifuged at 10000 x g for 1.5 minutes and the quantification of nucleic acid was measured using Nano Drop Lite spectophotometer (Thermocientific).

2. Reverse transcription

SuperScript VILO™ cDNA Synthesis Kit (Invitrogen, Grand Island, NY) was used to reverse transcribe the RNA samples. 200 ng of sample was mixed with 5x VILO reaction mix, 10x second strand (SS) enzyme mix and RNase-free water for a final concentration of 10 ng/μL of RNA. The samples were reverse transcribed using the DNA Engine DYAD (Peltier Thermal Cycler). The cycle consists of 10 minutes at 25°C, 1 hour at 50°C, 5 minutes at 85°C and a final temperature of 4°C.

3. RT PCR

Reverse transcribed RNA (cDNA) at a concentration of 10 ng was mixed with a master mix (TaqMan 2x universal PCR master mix) (Applied Biosystems, ROCHE), DNase free-water and the probes GAPDH (Hs99999905_m1), POU5F1 (Hs01654807_s1), SOX2 (Hs00602736_s1), KLF4 (Hs00358836_m1) and MYC (Hs00153408_m1) obtained from Applied Biosystems, for a total volume of 20 μL per well of a 96 well plate. The plate is quickly centrifuged and analyzed on 7500 Real Time PCR system (Applied Biosystems).

Minicircles Nucleofection

Nucleofection of T cells with minicircles were done with Amaxa Human T cell Nucleofector kit (LONZA, Allendale, NJ). Three to seven days after expansion 1-5×10^6 T cells were collected resuspended in 100 μL of nucleofector solution and mixed with 1-5 μg of DNA (Minicircles and Pmax control). The mix was transferred to a cuvette and inserted on the Nucleofector 2b device (LONZA, Allendale, NJ). The programs used were, T-023 and T-020. After nucleofection, 500 μL of pre-warmed T cell medium was added to the cuvette and the mix was transferred to a freshly coated anti-CD3 well.

Retrovirus infection

The day of infection depended on the cell diameter and the percent of CD3 positive cells in culture. At day 3 of expansion, 95% of the cells were CD3 positive and the average diameter was 10 μm. The day of infection the cells were harvested from culture and centrifuged at 1200 rpm for 5 minutes in a conical tube. The pellet was resuspended on T cell or T reg medium and cells counted with the hemocytometer. A total of 1×10^6 cells were reseeded on a well from a 6 well plate. 1 mL of each of the four Retrovirus (Oct4, Sox2, Klf4, and cMyc) were added to the wells. The Retrovirus was used fresh or frozen and un-concentrated. Protamine was also added to the well at a concentration of 8 μg/mL. In addition IL-2 cytokine was added at a concentration of 300 IU/mL. Anti-CD3 and anti-CD3/28 beads were added to the wells in some of the experiments. The cells received a spinfection, the 6 well plate was placed on the centrifuge for 1 hour at a speed of 2300 rpm at 32°C. After spinfecting the cells received a half media change (IL-2 was added to maintain the concentration of 300 IU/mL) and incubated overnight at 37°C, 5% CO_2. The next day the cells were harvested and centrifuged at 1200 rpm for 5 minutes. The pellet was resuspended in 0.5-1 mL of T cell or T reg medium and reseeded back on the same well. Virus, protamine and IL-2 were added in the same amounts as the day before. The cells were spinfected for a second time for 1 hour at 2300 rpm and half of medium volume was exchange afterwards. The cells were incubated at 37°C, CO_2. The next day cells were harvested and centrifuged in a conical tube for 5 minutes at 1200 rpm. The

supernatant containing the virus was discarded and the cell pellet resuspended in 2 mL of media with IL-2 at a concentration of 300 IU/mL and reseeded on the same wells. Cells were incubated for an additional 24-48 hrs. The next day cells were harvested and reseeded on iMEF feeders coated 6 well plates at a concentration of 1×10^5 cells per plate. Cells were incubated with T cell medium for 24-48 hrs and hES medium supplemented with 4 ng or 100 ng/mL of bFGF thereafter. The plates were cultured for 50 to 60 days.

Lentivirus infection (Tet-ON system)

Peripheral blood T lymphocytes at day 3 of expansion or T regulatory cells at day 3 of re-stimulation were harvested in a conical tube and centrifuged at 1200 rpm for 5 minutes. The cell pellet was resuspended in T cell medium and cells were counted in the ViCell to measure the cell diameter as well. A total of 1×10^5 cells per well were plated on a 12 well plate with 1 mL of medium containing IL-2 at a concentration of 300 IU/mL. Both viruses, 500 μL of the tetracycline transactivator and 500 μL of the tetracycline operator containing the four reprogramming factors were added to the well. Protamine was added at a concentration of 8 μg/mL. The plate was centrifuged at 1300 rpm for 1hr to spinfect the cells. After spinfection half of the medium volume was exchange and incubated at 37°C, 5% CO_2 for 24 hours. The next day cells were harvested in a conical tube and centrifuged at 1200 rpm for 5 minutes. Supernatant containing the virus was discarded and the cell pellet was re-suspended in 1 mL of medium containing IL-2 at a concentration of 300 IU/mL. Cells were reseeded in the same well and incubated for an additional 24 hrs at 37°C, 5% CO_2. 48 hrs after spinfection the cells were treated with doxycycline (a tetracycline derivative) to activate transcription of the four reprogramming factors (Oct4, Sox2, Klf4, and cMyc). Three different concentrations of doxycycline were used in the experiments: 0.5 μg/mL, 1 μg/mL, and 2 μg/mL. 48-72 hours post-spinfection the cells were reseeded on irradiated MEF feeders coated plates with T cells media. 24-48 hours later medium was changed to hES medium containing 4 ng/mL or 100 ng/mL of bFGF. Doxycycline was added the same day medium was exchanged for 15 days post-infection. The plates were kept in culture for 50 to 60 days post-infection and medium was changed every other day.

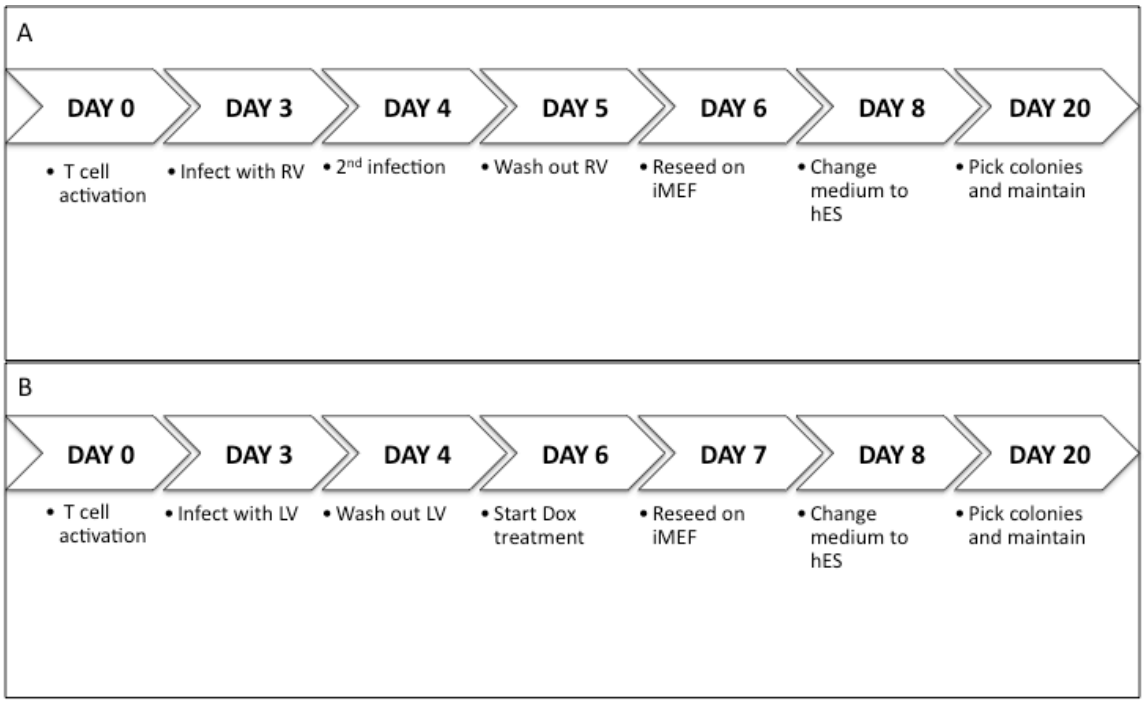


Figure 1. Timeline of Retrovirus and Lentivirus infection. (A) Retrovirus infection. (B) Lentivirus infection. LV, lentiviral vector; iMEF, irradiated mouse embryonic fibroblasts. RV, retroviral vector; Dox, doxycycline.

Sendai virus infection

CytoTune^{TM} – iPS Sendai Reprogramming Kit (Life Technologies) was used for the Sendai virus experiments. Peripheral blood T cells or peripheral blood T regulatory cells were activated and expanded following the above protocol. At day 4 of expansion the cells were collected and centrifuged for 5 minutes at 200 x g. The supernatant was removed and the pellet resuspended in 1 mL of medium (Xvivo for T regs and T cell medium for T cells and memory cells). Cells were counted in the Vi-CELL Cell Viability Analyzer (Beckman Coulter) and plated on a 12 well plate with 1 mL of medium containing IL-2. T cells were plated on freshly coated anti-CD3 wells and T regulatory cells with anti-CD3 and anti-CD28 coated beads. The plate was incubated for 24 hours. The next day, the four Sendai virus vectors were added to the culture individually, each at an MOI of 5 or 20. The plate was incubated for an additional 24 hours. The next day,

cells were collected from the wells and centrifuged at 200 x g for 5 minutes to remove the virus, resuspended on T cell medium containing IL-2 and re-plated in the same wells for an additional 24 hours. At 48 hours post infection the cells were collected, centrifuged and counted. 5×10^4 and 5×10^5 cells resuspended in T cell or X vivo medium were reseeded in a 100-mm dish coated with irradiated mouse embryonic fibroblasts (iMEFs) feeder cells and incubated in 37C for 24 hrs. The next day medium was changed to hES containing 4 ng/mL of bFGF.

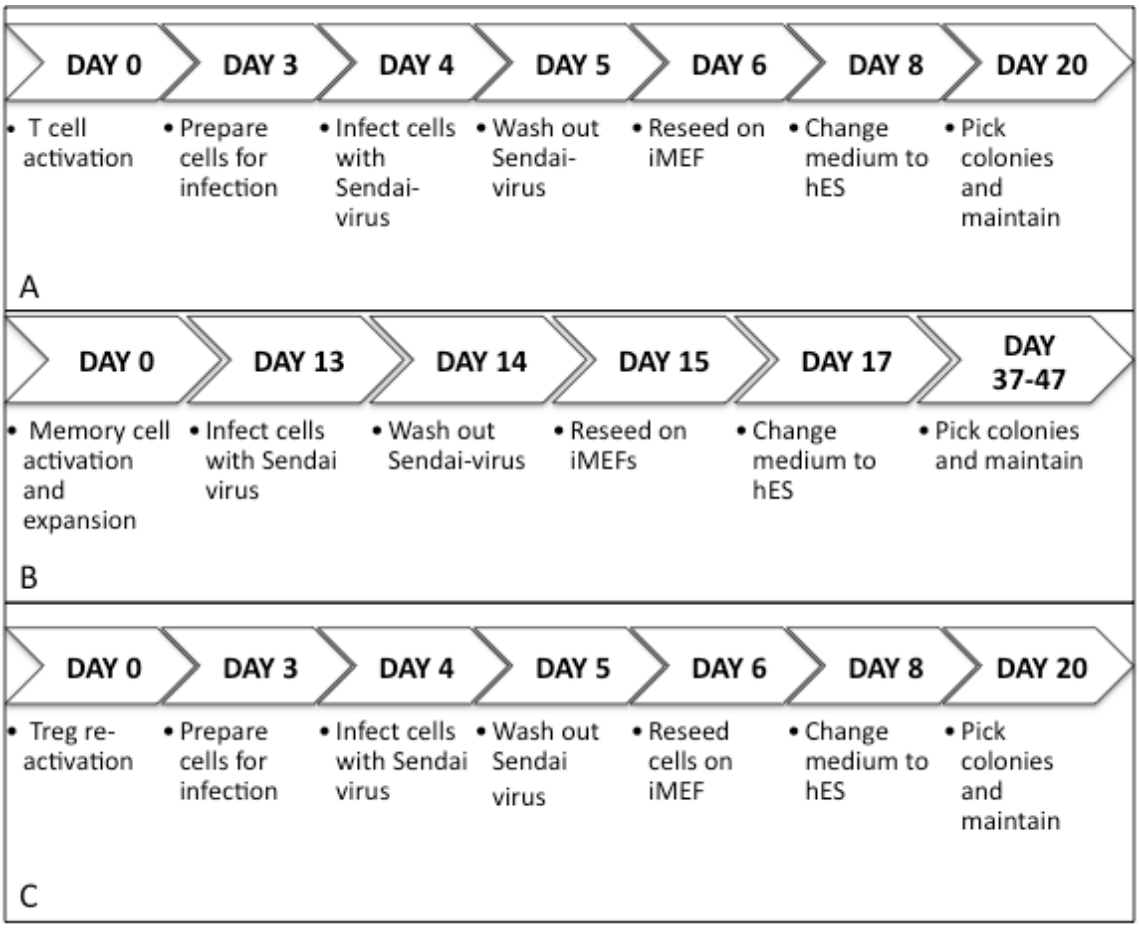


Figure 2. Timeline of Sendai virus infections. (A) Peripheral blood T cells. (B) Memory T cells. (C) Regulatory T cells. iMEF, irradiated mouse embryonic fibroblasts.

iPSC culture and characterization

iPSC characterization adapted from Tolar *et al.,* 2011a; Tolar *et al.,*2011b.

1. iPSC culture

Approximately 20-30 days after infection iPSC-like colonies appeared. The flat, translucent, round-edged colonies were manually picked using a pasteur pipette. An individual colony was cut into 5-6 pieces and transferred to a freshly coated iMEF plate containing hES media. Plates were incubated at 37°C with 5% CO_2 and media was changed daily. Colonies were picked manually using 1x collagenase (Invitrogen) every 5-7 days and were transferred to a freshly coated iMEF plate.

2. Immunofluorescence and alkaline phosphate staining of iPSC

The iPSC were grown on 12 well plates containing iMEFS for 5-7 days and fixed with 4% paraformaldehyde (PFA) for 20 minutes. Cells were treated with 0.2% TritonX (Sigma) in PBS for 30 minutes for nuclear permeation and blocked in 3% BSA in PBS for 2 hours. Cells were incubated with the primary antibody overnight at 4°C. The following antibodies were used: TRA1-60 (MAB4360, 1:400), TRA1-81 (MAB4381, 1:400), SSEA4 (MAB4304, 1:100) and SSEA3 (MAB-4303, 1:100 (all from Chemicon)), NANOG (EB06860, 1:100 (Everest)), OCT3/4 (AB27985, 1:200 (ABCAM)), SOX2 (630802, 1:500 (Biolegend)). The next day cells were washed twice with PBS and secondary antibodies were added to the wells for 1 hour at room temperature. All secondary antibodies were the Alexa Fluor Series from Invitrogen and all diluted 1:500. Three drops of 4',6-diamidino-2-phenylindole (DAPI) was added to the wells and images were taken with fluorescence microscope (Olympus Flowview 500 confocal microscopy, Center Valley, PA). Alkaline phosphatase activity was analyzed using alkaline phosphatase staining kit following manufacturer's recommendations (Millipore).

3. Intravital staining

TRA-1-60 antibody (Millipore, 1:400) and secondary antibody Alexa 488–conjugated anti–mouse IgM (Invitrogen, 1:400) were diluted in hES medium and added into the culture plate. The plate was incubated in 37°C for 1 hour. TRA-1-60^{+} colonies were identified under a fluorescence microscope.

4. Gene expression analysis

Total RNA extraction of iPSC colonies and reverse transcription was done as described above. Reverse transcribed RNA (cDNA) at a concentration of 10 ng was mixed with a master mix (TaqMan 2x universal PCR master mix) (Applied Biosystems, ROCHE), DNase free-water and the probes GAPDH (Hs99999905_m1), POU5F1 (Hs01654807_s1), SOX2 (Hs00602736_s1), KLF4 (Hs00358836_m1), MYC (Hs00153408_m1), NANOG (Hs 02387400_g1), Lin28 (Hs 00702808_s1), Rex01, ABCG2, DNMT3b(Hs01003405_m1) obtained from Applied Biosystems, for a total volume of 20 μL per well of a 96 well plate. The plate is quickly centrifuged and analyzed using 7500 Real Time PCR system (Applied Biosystems).

5. Bisulfite genomic sequencing

Genomic DNA was isolated using PureLink Genomic DNA Mini Kit (Invitrogen) following manufacturers protocol. Bisulfite treatment was done using EpiTect Bisulfite Kit (Qiagen, Valencia, CA). Treated DNA was amplified with PCR using OCT4 and NANOG specific primers (Freberg *et al*., 2007). PCR products were gel purified using PureLink Quick Gel Extraction and PCR Purification Combo Kit (Invitrogen) and cloned into bacteria with TOPO TA Cloning Kit for sequencing (Invitrogen).

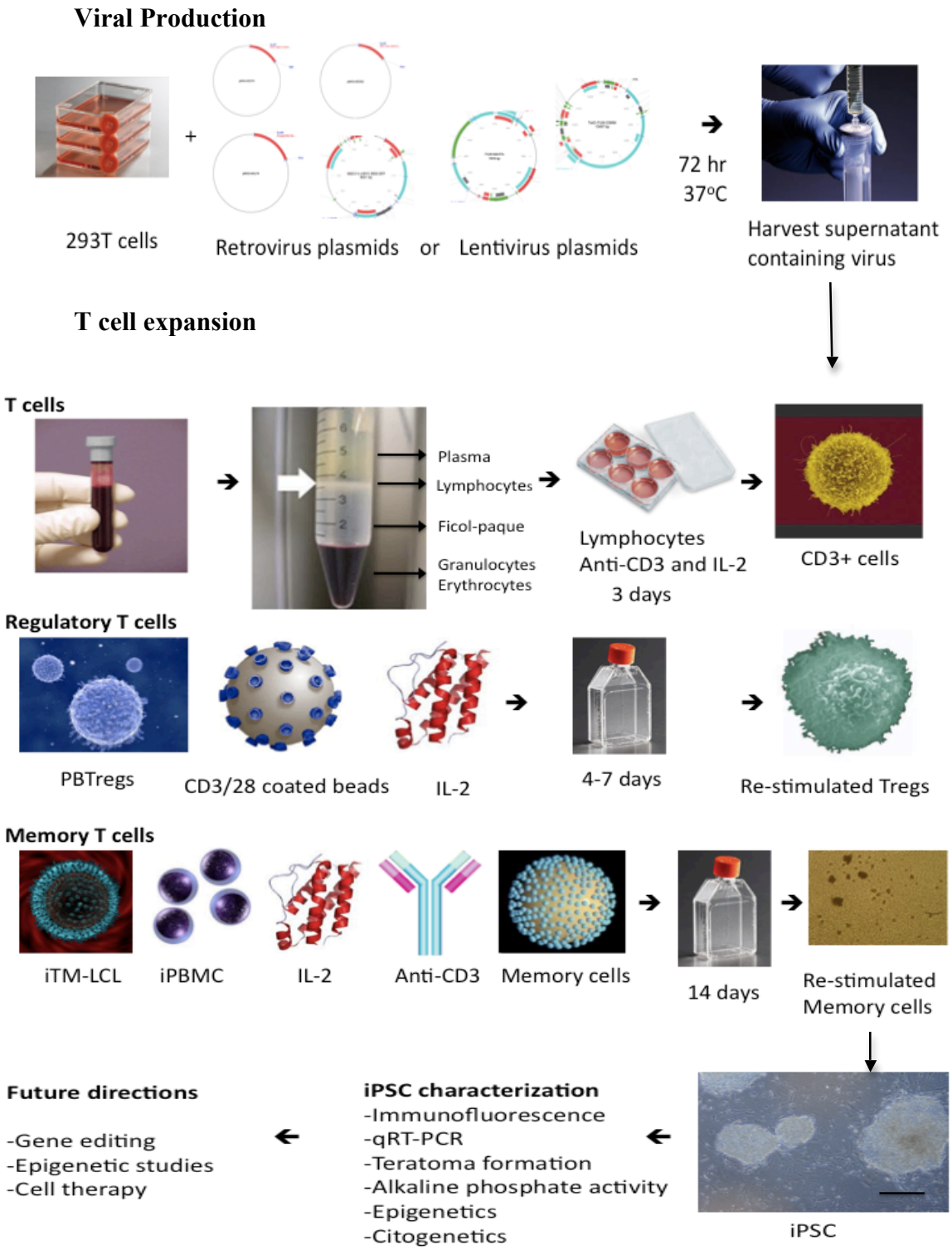


Figure 3. Summary scheme of methods and future experiments. Bar scale- 0.25 mm.

RESULTS

To determine the optimal reprogramming platform for effector and central memory T cells we tested 4 different vectors: retrovirus, lentivirus, Minicircle, and Sendai virus. Whole T cells and T reg cells from peripheral blood were transduced with each vector (summarized in Table 1). Efficacy of transduction was measured by flow cytometry, qRT-PCR and iPSC formation. Here, we show the data from these experiments that lead us to determine the most favorable platform for T cell reprogramming. We also show data from optimization experiments done for some of the viral vectors.

Table 1 | **Summary of reprogramming experiments**

Cell type	Vector	Amount
Peripheral blood T cells	Minicircle	1, 2, and 5 μg per 1-5×10^6 cells
	Retrovirus	MOI of 1-5
	Lentivirus	MOI > 5
	Sendai virus	MOI of 5 MOI of 20
Peripheral blood Regulatory T cells	Retrovirus	MOI of 1-5
	Lentivirus	MOI > 5
	Sendai virus	MOI of 5
Effector memory T cells	Sendai virus	MOI of 20
Central memory T cells	Sendai virus	MOI of 20

Reprogramming peripheral blood T cells to iPSC

1. Reprogramming with Retroviral vector

Peripheral blood T cell iPSC have been derived previously with the use of retrovirus (Brown *et al*., 2010). We used this as a model for our retrovirus experiments. Peripheral blood T cells were activated and expanded from PBMCs for a minimum of 3 and up to 7

days. At day 3, 94.97% of the cells were CD3 positive (Figure 4) assuring infection of the desired population of cells and a low possibility of infecting undesired PBMC (B cells, NK, etc). Infections at day 3 through day 7 showed that cells were more viable post infection when infected at an earlier time, thus we decided to infect the T cells at day 3 or 4 from then on. We also measured the diameter of the cells during activation as a guide for T cell activation. T cell proliferation involves signal transductions through the TCR and cytokines (e.g. IL-2) as a secondary signal to progress in activation. The combination of both signals involves phosphorylation of tyrosine in intracellular proteins leading to activation of genes involved in activation and proliferation. The activation of gene transcription is associated with an increase in cell size (Ahuja *et al.,* 1993). As shown in Figure 5 the cells grow in size from day 3 through day 6 where they start to decrease in size. This data is consistent with the viability of cells after infection, whereas T cells infected during growth are more viable than T cells infected at their growth peak.

In our experiments two rounds of retrovirus infection were done and 24 to 48 hours post the second infection T cells were reseeded on iMEFs. bFGF at higher concentrations have been known to help iPSC derivation and maintenance (Varum *et al.,* 2009), thus, bFGF at a concentration of 100 ng/mL was used versus 10 ng/mL used routinely in our lab. During our experiments, both bFGF concentrations were used, however, no difference in iPSC generation was seen. MEF conditioned medium was used as culture medium after reseeding virus treated cells on iMEFs as a new approach to our experiments. MEF conditioned medium is usually used when growing iPSC on feeder free conditions to deliver MEF secreted products to the culture which are important for iPSC formation. We used the MEF conditioned medium in addition to the feeder layer on our cultures to see if an excess of MEF secreted products increase the formation of iPSC. Although Brown *et al*. showed successful reprogramming of peripheral blood T cells with retroviral vectors, our platform was not optimal to derive T cell-iPSC. This inefficiency can be related to the retroviral vectors used to carry the genes of interest. Brown *et al.* used MoMuLV based retroviral vector to carry the four reprogramming genes (OSKM). We used a p-MIG based retroviral vector as our cargo to deliver the four transgenes. Variegation of

retroviral vectors can account for the efficiency of transduction and gene expression (Zentilin *et al.,* 2000), thus suggesting and explaination for the different observations in our study.

We analyzed T cells during the reprogramming process for green fluorescent protein (GFP) and transgene expression to assure viral transduction. GFP expression is an indirect marker for transduction. Each of the four retroviruses used contain the gene of interest followed by an internal ribosome entry site (IRES) and GFP marker (Park *et al*., 2008). Flow cytometry analysis on T cells infected with retrovirus at day 2 post-infection shows GFP expression suggested that the virus was infecting the cells and there was integration of transgenes into the cell genome. However, qRT-PCR for OSKM expression showed different results; Oct4, Sox2 and c-Myc show a maximum of 10 fold increase on the levels of transcript compared to a T cell control whereas a 1000 fold increase was seen in the Klf4 transcript (Figure 6). To see whether or not high Klf4 expression after viral infection is a T cell specific phenomenon we infected human fibroblast with all four retrovirus and isolated total RNA 48 hours after infection. qRT-PCR showed lower expression of Klf4 on human fibroblasts compared to that seen on T cells suggesting that up-regulation of Klf4 transcripts after virus transduction is T cell phenomenon (Figure 7). To investigate if Klf4 levels were viral specific, we infected T cells with only Oct4, Sox2 and cMyc retrovirus. qRT-PCR analysis showed no expression of Klf4 when T cells are not infected with retrovirus containing the Klf4 gene, suggesting that up-regulation of Klf4 is viral specific (Figure 8). Nonetheless, it is important to note that the probes used for qRT-PCR do not differentiate between exogenous or endogenous OSKM genes, thus, Klf4 transcript up-regulation could be from either the endogenous or exogenous genes. Endogenous Klf4 expression is seen in activated T cells when cultured *in vitro* and it binds to the IL17a promoter. IL17a, drives differentiation of naïve T cells to Th-17 cells. Thus, Klf4 plays an important role in T cell development and differentiation (An *et al.,* 2011). The fact that Klf4 participates in T cell activation pathways could account for the un-efficiency of reprogramming T cells in some of our studies.

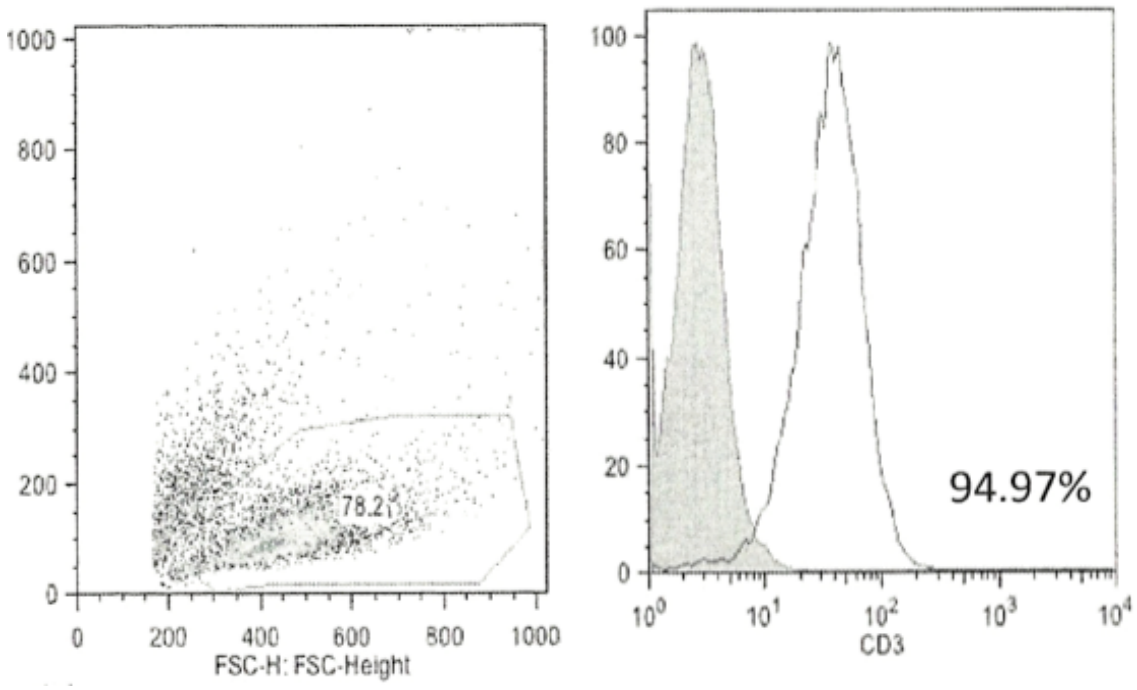


Figure 4. CD3 positive cells after T cell expansion. Flow cytometry analysis in T cells at day 3 of expansion with anti-CD3 and IL-2.

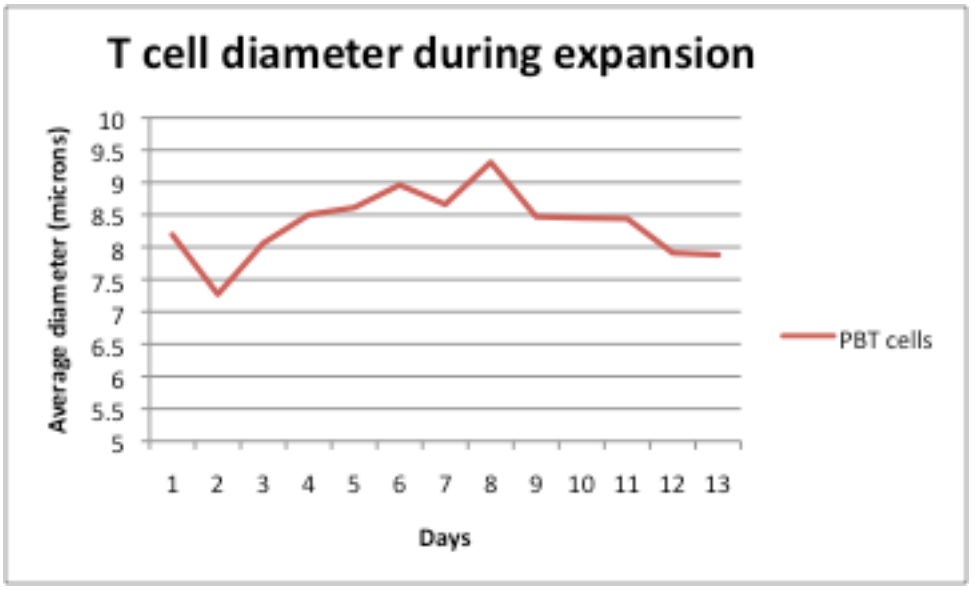


Figure 5. T cell diameter during expansion. Peripheral blood T cells during expansion measured daily for 13 days.

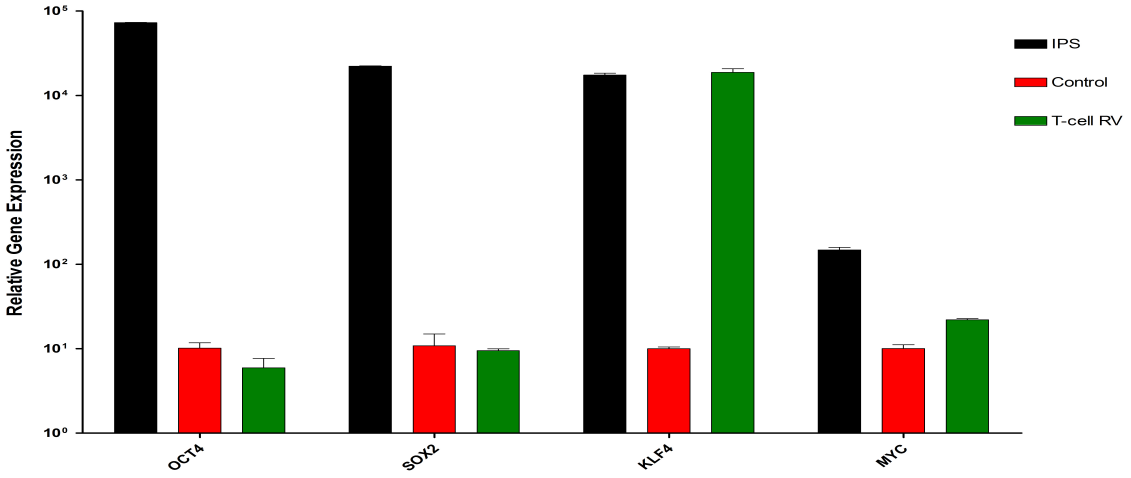


Figure 6. Gene expression analysis in T cells infected with Retrovirus. Quantitative RT-PCR analysis of Oct4, Sox2, Klf4 and cMyc genes in T cells 48 hours post Retrovirus infection. Expression levels are relative to a T cell control (T cells without viral infection). All values were normalized to the endogenous GAPDH expression. iPS, induced pluripotent stem cells; Control, T cell control; RV, retroviral vector; Tcell RV, Tcell infected with retroviral vectors.

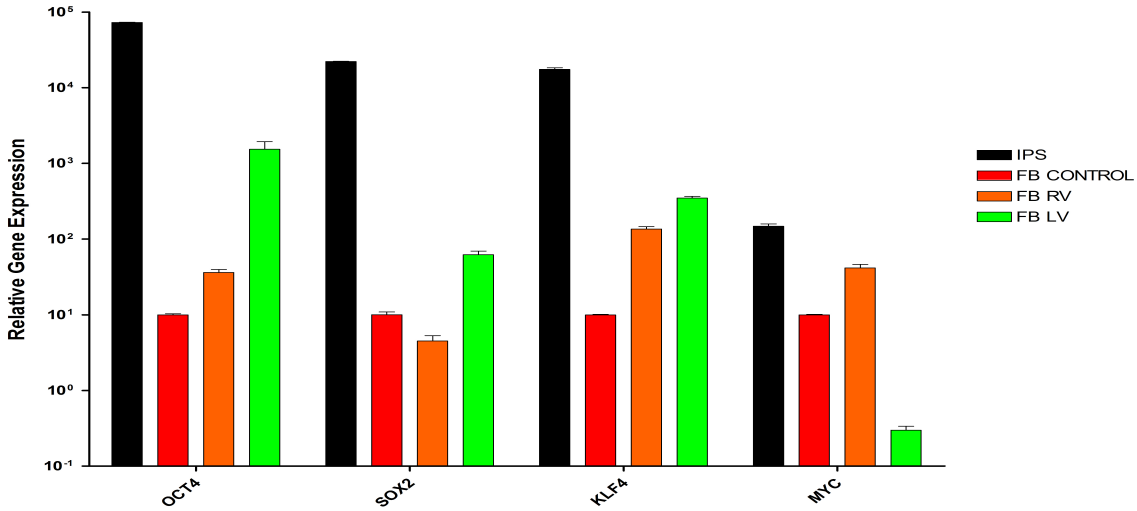


Figure 7. Gene expression analysis in human fibroblasts. Quantitative expression analysis of Oct4, Sox2, Klf4, and cMyc in human fibroblasts 48 hours after retroviral infection (orange bar) and doxycycline treated fibroblasts 24 hours after lentiviral infection (green bar). Gene expression is relative to the human fibroblast control (untreated fibroblast, red bar). All values were normalized to endogenous GAPDH expression. iPS, induced pluripotent stem cells; FB, fibroblast; RV, retroviral vector; LV, lentiviral vector.

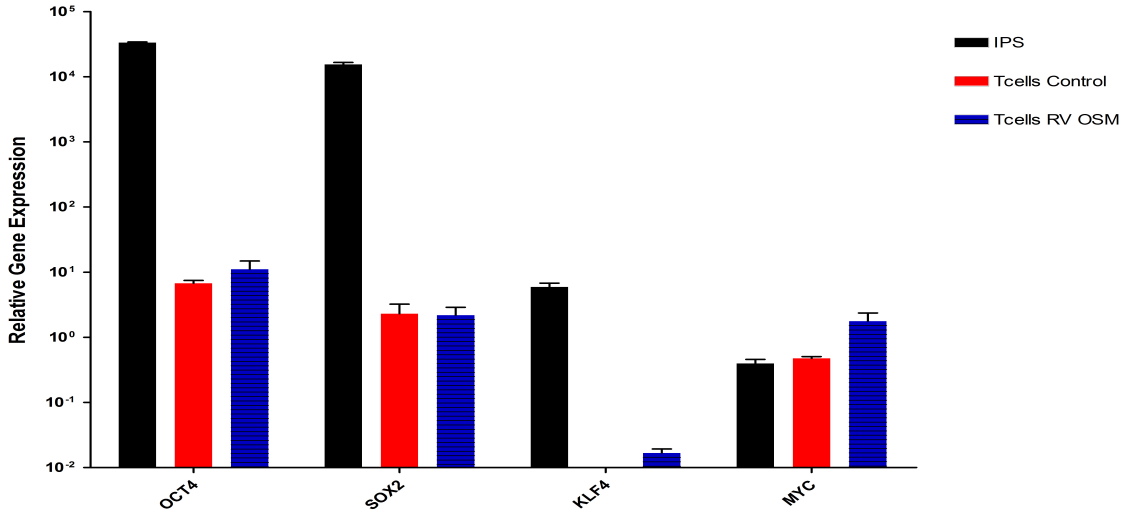


Figure 8. Gene expression analysis in T cells infected with only 3 factors. Quantitative RT-PCR of T cells infected with Retrovirus containing Oct4, Sox2 and cMyc (blue bar). Klf4 was not introduced. Gene expression is relative to untreated T cells (T cell control, red bar). iPS, induced pluripotent stem cells; RV, retroviral vector; OSM, Oct4, Sox2, c-Myc.

2. Reprogramming with Lentiviral vector

Lentiviral vectors are more efficient than retroviral vector at infecting a variety of somatic cells and they increase reprogramming efficiency when used to express polycistronic cassettes in which a single promoter directs the synthesis of all genes in the cassett (Carey *et al.,* 2009; Sommer *et al.,* 2009; Stadtfeld and Hochedlinger, 2010). As an alternative to the retroviral vectors used in our previous experiments, we used a doxycycline inducible polycistronic lentiviral vector. This construct contains a gene that encodes for a tetracycline transactivator protein in one vector and in another one, genes encoding for all four reprogramming factors OSKM, driven by a single promoter, which is activated by the transactivator in the presence of doxycycline (a tetracycline derivative) (Carey *et al.,* 2009; Hockemeyer *et al.,* 2008). We hypothesized that manipulating the gene expression or silencing of single polycistronic cassette with all four reprogramming factors will enhance OSKM expression on T cells and subsequently derivation of T cell-

iPSC. Fibroblasts and PBMCs have been successfully reprogrammed with a similar construct (Sommer *et al*., 2009; Staerk *et al*., 2010). Moreover, iPSC derived from PBMCs showed VDJ recombination, suggesting that T cells were fully reprogrammed to iPSC using this approach. This vector does not have a marker protein (e.g. GFP) to titer or test for successful viral infection with flow cytometry as we did with the retrovirus. Thus, our first experiment was to determine the optimal concentration of virus to infect T cells by analyzing the Oct4, Sox2, Klf4 and cMyc (OSKM) gene expression with qRT-PCR. We infected T cells at different concentrations with different volumes of concentrated lentiviral vector. Cells were treated with doxycycline (0.5 μg/mL) after 24-48 hours post-infection. We proceeded to isolate total RNA 48 hours post doxycyline treatment followed by qRT-PCR to analyze expression of Oct4, Sox2, cMyc, and Klf4 (OSKM) (Figure 9). The highest OSKM expression was seen with 1.0×10^5 cells infected with 500 μL of virus. We used these concentrations for all the proceeding lentiviral experiments.

To determine the optimal expression profile for reprogramming we proceeded to compare gene expression levels of T cells infected with retroviral vectors versus lentiviral (Figure 10). We infected T cells with 1 or 2 rounds of lentivirus or retrovirus and compared the gene expression levels by qRT-PCR depicted in figure. The data shows that both retrovirus and lentivirus produce similar gene expression levels in T cells. Even though, lentiviral vectors have been more efficient in reprogramming somatic cells, both retroviral and lentiviral vectors are integrating vectors and they work in the same fashion. This similarity could explain why their expression levels are similar in T cells. However, other factors can contribute to gene expression and transduction efficacy. For example, gene expression can be directly affected by doxycycline dose in the lentiviral construct (Vogel *et al.,* 2004). Also the stability of iPSC can be donor dependent, suggesting that gene expression can differ between donors (Vitale *et al.,* 2012).

To test the efficacy of transgene expression between different donors we activated T cells from 2 different donors and treated with 2 rounds of lentiviral infection. Doxycycline (1

μg/mL) was added to the culture medium 48 hrs post infection and cells were reseeded on iMEFs. qRT-PCR analysis 48 hrs post doxycycline treatment show a 10 fold increase on the levels of Oct4 and Sox2 transcripts, a 100 fold increase on the levels of Klf4 and no increase on cMyc transcript when compared to a T cell control. Also, no significant difference was seen between donors and the rounds of viral infections (Figure 11).

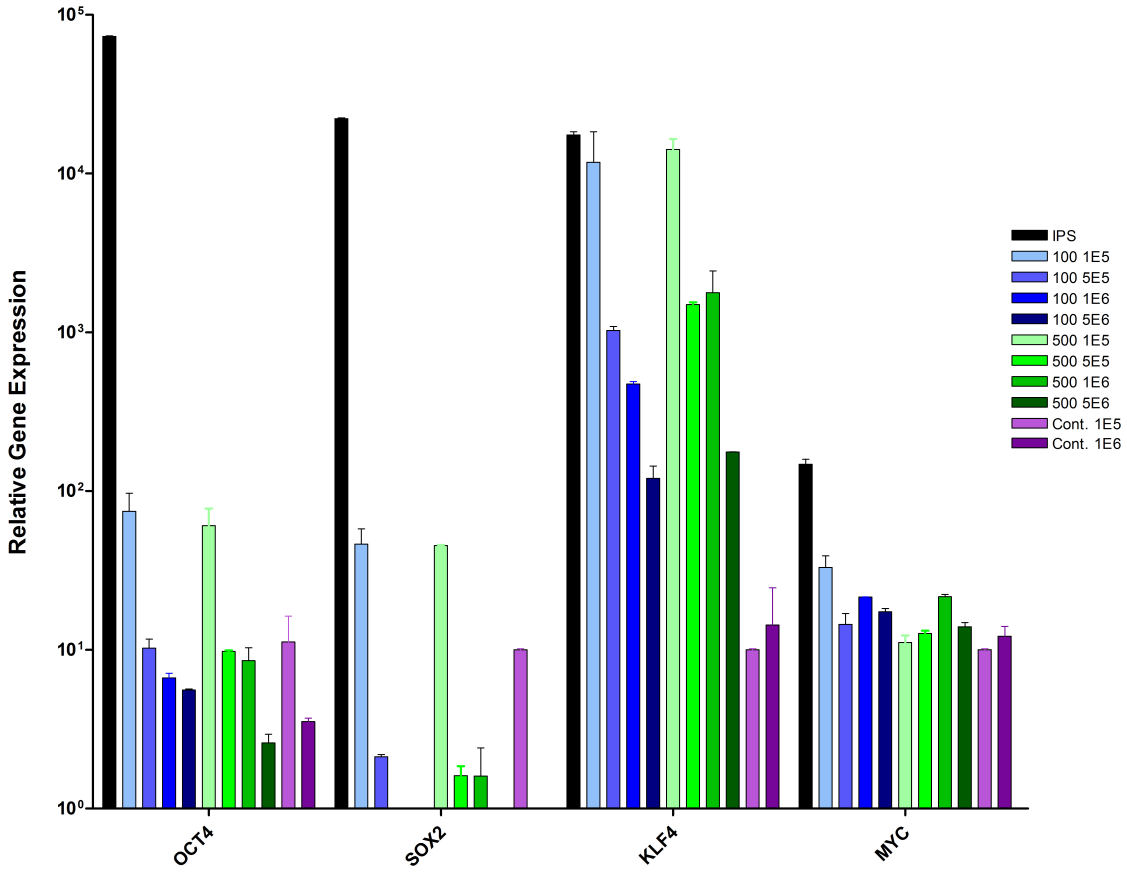


Figure 9. Gene expression analysis in T cells infected with Lentivirus. Quantitative RT-PCR analysis of Oct4, Sox2, Klf4 and cMyc on different concentrations of T cells infected with different volumes of concentrated Lentivirus. Cells were treated with doxycycline 48 hours prior to analysis. Blue bars show different concentrations of T cells treated with 100 μL of Lentivirus. Green bars show different number of T cells treated with 500 μL of Lentivirus. Gene expression is relative to normal untreated T cells (purple bars). iPS, induced pluripotent stem cells; Cont., Control; 100, 100 μL; 500, 500 μL.

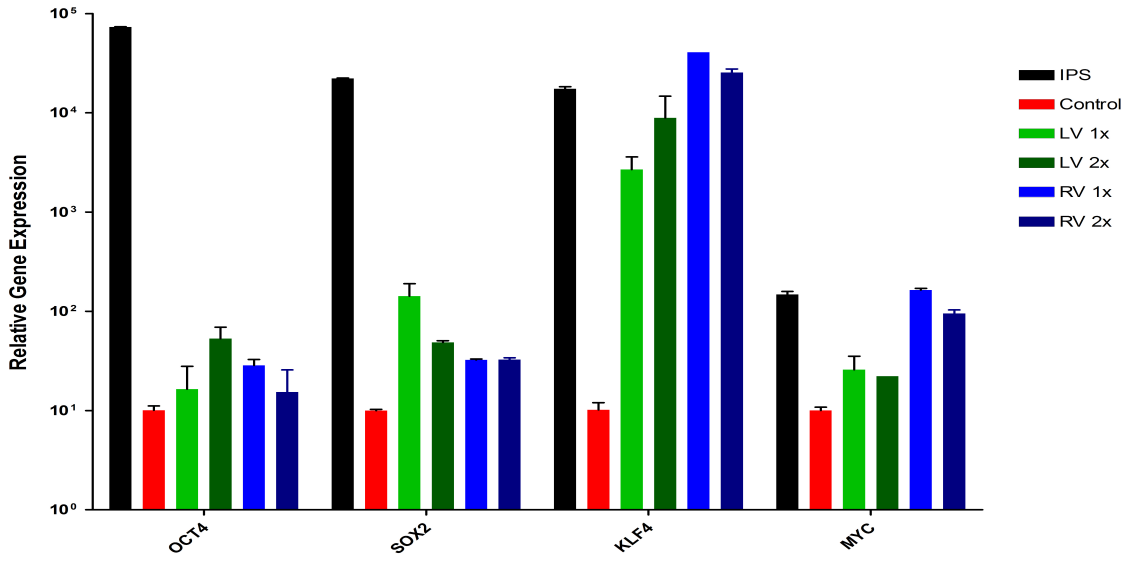


Figure 10. Gene expression analysis in T cells infected with Retrovirus and Lentivirus. Quantitative RT-PCR analysis of Oct4, Sox2, Klf4 and cMyc expression levels in Doxycycline treated (1 μg/mL)T cells infected once or twice with Lentivirus (LV 1x and LV 2x) and T cells infected once or twice with Retrovirus (RV 1x and RV 2x). Gene expression is relative to untreated T cells (control). iPS, induced pluripotent stem cells; LV, lentiviral vector; RV, retroviral vector; 1x, one infection; 2x, two infections.

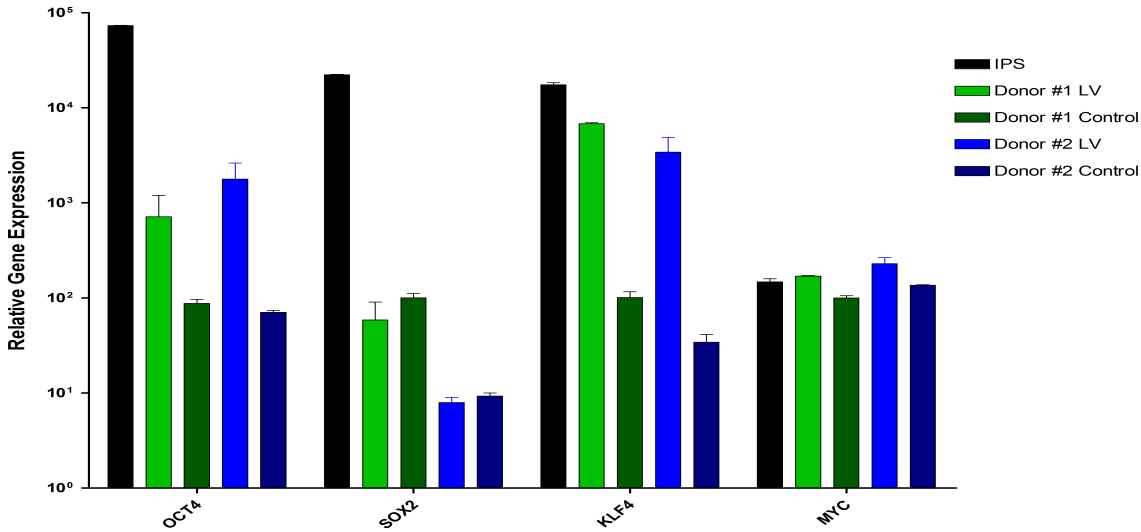


Figure 11. Gene expression in T cells from two different donors. Quantitative RT-PCR analysis of Oct4, Sox2, Klf4, and cMyc transcripts in doxycycline treated T cells (1 μg/mL) infected with lentiviral vector. Gene expression of infected T cells from donor #1 and donor #2 are relative to untreated T cells from the same donor (control). iPS, induced pluripotent stem cells; LV, lentiviral vector; RV, retroviral vector.

3. Reprogramming with episomal vector (minicircle)

Permissive conditons for iPSC formation was not achieved with the use of retroviral and lentiviral vectors. To this end we tested the minicircle vector for the production of viral free-T cell-iPSC. Lentiviral and retroviral vectors integrate into cell genome, potentially causing insertional mutagenesis. In addition they can reactivate expression of viral genes in iPSC, these could induce tumors in mice and truncate the differentiation potential of iPSC *in vitro* (Yee, 2010). Thus, non-integrating vectors (e.g. minicircle) are preferred for clinical applications. The minicircle vector contains four reprogramming factors, Oct4, Sox2, Nanog and Lin28, which are known to reprogram human somatic cells in the same manner as "Yamanaka's factors" (Yu *et al.,* 2007). This vector has been shown to reprogram adipose tissue to generate transgene-free iPSC (Fangjun *et al*., 2010; Narsinh *et al.,* 2011). However, it has not been reported to date with suspension cells. In our experiment, 1×10^6 T cells at day 5 to 7 of expansion were nucleofected with different concentrations of minicircle vector. To determine the efficiency of nucleofection, we analyzed GFP expression 48 hours post nucleofection by flow cytometry. Transfection efficiency was of 17% (Figure 12), yet the viability and cell numbers were not optimal for subsequent infection via lipofection as the protocol suggests (Amaxa Human T cell Nucleofector kit). To optimize nucleofection conditions, a higher concentration of cells (5×10^6) and 2 different nucleofector programs (T-020 and T-023) were used. However, there was no difference on the GFP expression compared to the first experiment (data not shown). We concluded that this platform was not optimal for T cell reprogramming due to low viability of cells after nucleofection and low transfection efficiency after nucleofection.

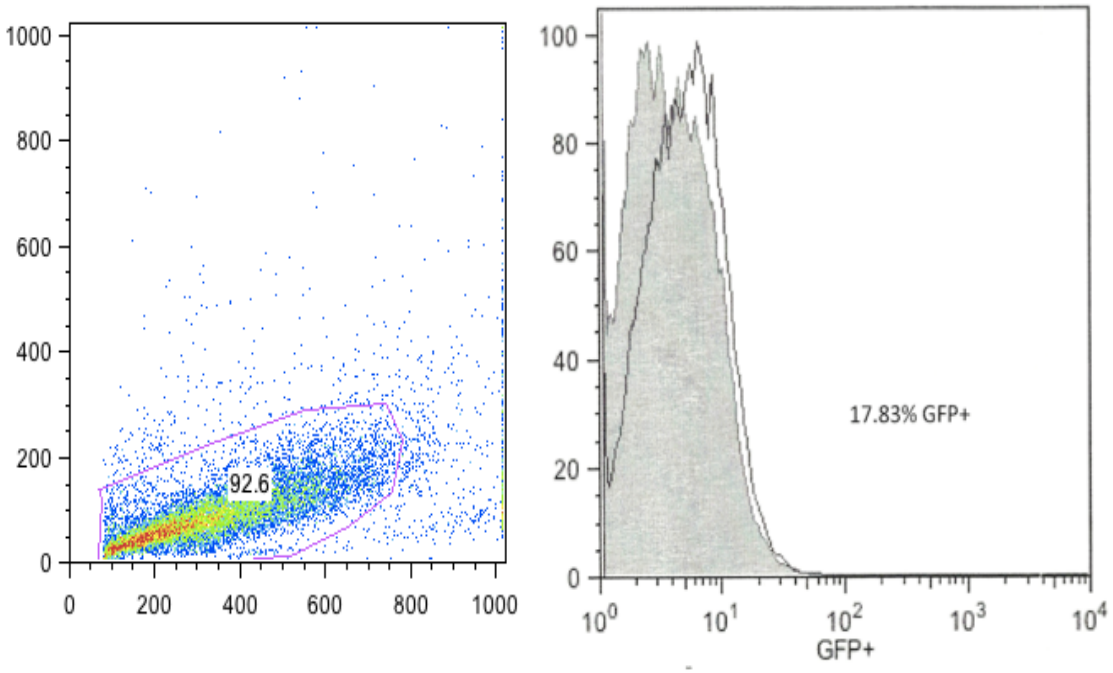


Figure 12. GFP expression in peripheral blood T cells nucleofected with minicircle. Flow cytometry analysis for GFP expression in T cells nucleofected with 5 μg of minicircle DNA. GFP, green fluorescent protein.

4. Reprogramming with Sendai virus vector

Sendai virus vectors provides an efficient way to develop viral free iPSC. The Sendai virus vector used in our experiments is a temperature sensitive vector, thus providing a non-invasive approach to remove any residual viral products after deriving iPSC (Ban *et al.,* 2011; Okano *et al.,* 2003; Seki *et al.,* 2010). T cells at day 3 of expansion were infected with Sendai virus at an MOI of 5 and reseeded on iMEF plates. After 30 days after infection, no iPSC like colonies were seen. The efficiency of Tcell-iPSC formation has been shown to directly correlate with the MOI (Seki *et al*., 2011). For our next experiment we infected T cells at an MOI of 20. Ten days post infection, iPSC-like colonies emerged on iMEF plates. Colonies were picked based on their appearance at 15 to 20 days post infection and maintained in culture by passaging every 5 to 7 days. After

passage 6, 12 out of 21 clones picked were viable and 2 clones were chosen for detailed characterization of iPSC specific parameters.

Tcell-iPSC expressed surface markers as would be expected from a true iPSC: Tra-1-81, Tra-1-61, stage-specific embryonic antigens- 3 and -4, OCT4 and NANOG. Tcell-iPSC also showed alkaline phosphatase activity and mRNA expression consistent with pluripotency (such as OCT4, NANOG, LIN28 and DNMT3b) (Figure 13). During the reprogramming process it is believed that epigenetic changes are induced by the reprogramming factors (Aasen *et al.,* 2008; Park *et al.,* 2008; Santos and Dean, 2004). Thus, we used bisulfite sequence to analyze the status of the OCT4 and NANOG promoters in Tcell-iPSC. A methylation pattern is expected from a mature or differentiated cell, indicating silencing of pluripotent genes. On the other hand, iPSC are expected to have an unmethylated pattern indicating gene expression. Tcell-iPSC clones show methylation patterns characteristic of iPSC, whereas the T cells show methylation on the pluripotent gene promoter, as expected (Figure 14). To confirm the origin of the iPSC donor T cells and a clone of the resultant iPSC were typed for the human leukocyte antigen (HLA) Class I (A, B, and C alleles) and Class II (DR and DQ alleles). Both, donor T cells and Tcell-iPSC showed the same HLA type, confirming that the resultant iPSC were indeed derived from donor cells and they retain the genetic identity of the donor as well. Dr. Bow from Hartford Hospital (Harford, CT) kindly did the HLA typing assay and provided us the data (data not shown). All together, the gene expression and phenotype of Tcell-iPSC are consistent with successful reprogramming events. Our findings confirm previous studies, in the formation of Tcell-iPSC and the correlation between iPSC formation and MOI (Seki *et al.,* 2011). We concluded that the Sendai virus vector was the optimal platform for deriving Tcell-iPSC and the best approach to derive iPSC from memory T cells.

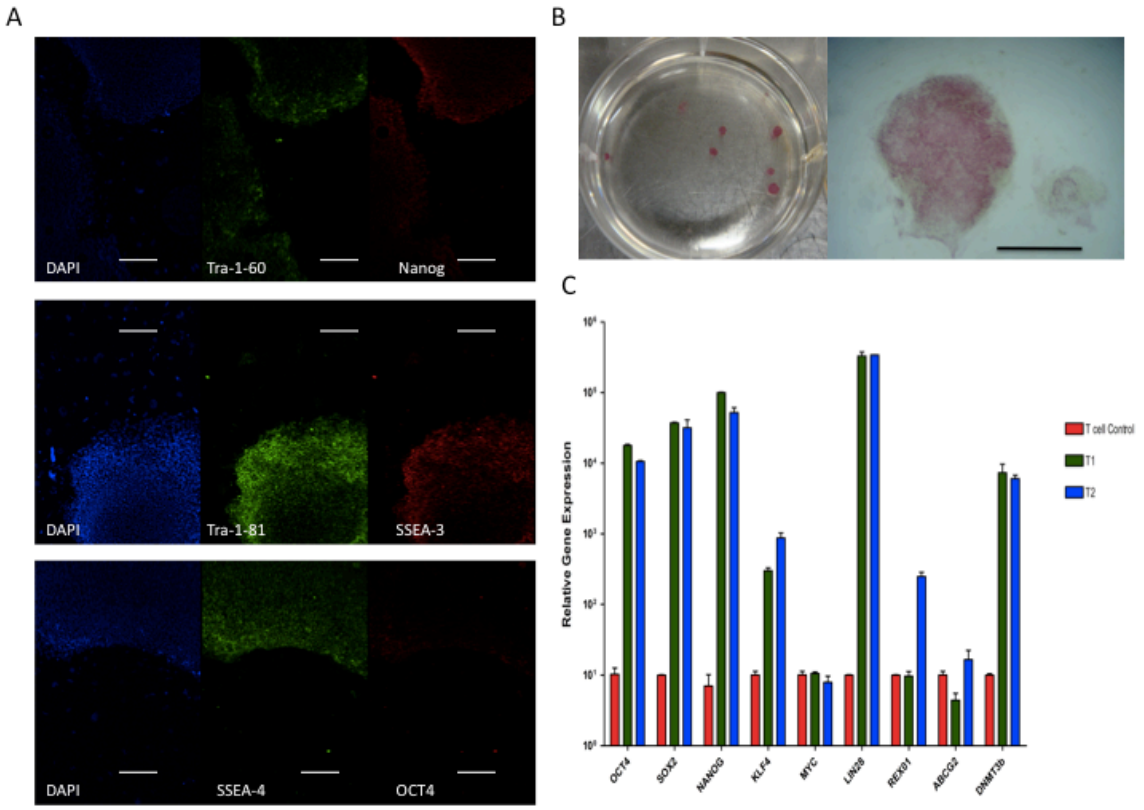


Figure 13. Induction of peripheral blood T cells into iPSC with Sendai virus. (A) Immunostaining with Tra-1-81, Tra-1-61, SSEA-3 and -4, Nanog, and Oct3/4. Also iPSC stained with, 4,6-diamidino-2-phenylindole (DAPI) showing nuclei of individual cells in colonies. Bar scale is 50 μm. (B) Alkaline phosphatase stain. Bar scale is 0.5 mm. (C) Protein expression profile of T cell iPSC. Quantitative RT-PCR analysis of Oct4, Sox2, Nanog, Klf4, Myc, Lin28, Rex01, ABCG2, and DNMT3b expression levels in 2 different T cell iPSC clones (T1 and T2). Gene expression is relative to normal untreated T cells (control). All values are normalized against endogenous GAPDH expression. iPS, induce pluripotent stem cell; T1, Tcell-iPSC clone #1; T2, Tcell-iPSC clone #2.

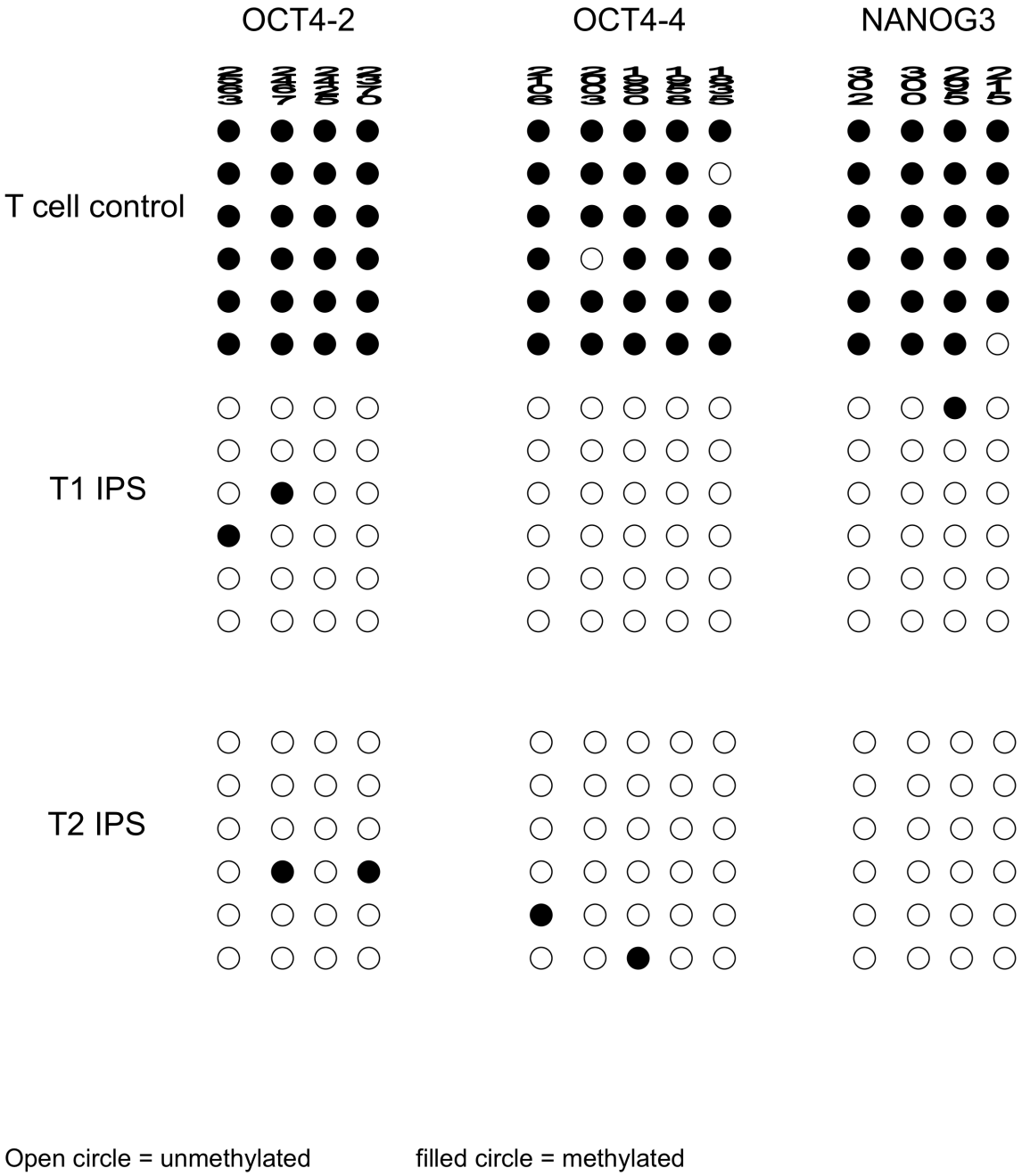


Figure 14. Epigenetic profile of peripheral blood T cell iPS cells. Bisulfite sequencing of the Oct4 and Nanog promoters in T cell control and two Tcell-iPSC clones. Open circles indicate unmethylated CpGs and filled circles indicate methylated CpGs. Each row of circles denote the sequencing reaction of a specific amplicon. Each column represents the CpG position. iPS, induced pluripotent stem cells; T1, Tcell-iPSC clone #1; T2, Tcell-iPSC clone #2.

Reprogramming regulatory T cells to iPSC

1. Reprogramming with Retroviral and Lentiviral vector

Our first approach to reprogram T regs was with the use of integrating vectors. We re-stimulated peripheral blood T regs and measured the activation status by cell diameter. During activation, T reg cell diameter changes from an average of 7 μm to 10 μm (this data was provided by Dr. Hippen's laboratory (UMN)). At this point, T regs were infected with either retrovirus or lentivirus. Recalling the aberrant levels of Klf4 expression produced by peripheral blood T cells, we tested T regs in this matter by retroviral infection of 3 factors (Oct4, Sox2, cMyc) as well as all 4 factors (Oct4, Sox2, Klf4, cMyc). T regs infected with lentivirus were treated with 1 μg/mL of doxycycline 24 hours after infection. To test the viral transfection efficiency, we tested for OSKM expression by qRT-PCR. Surprisingly, T regs infected with all 4 retroviruses show lower Klf4 levels compared to previous analysis on peripheral blood T cells (10^2 versus 10^4 respectively). However, when Klf4 retrovirus was not given to T regs no expression of Klf4 transcript was seen. This data suggests that endogenous or exogenous Klf4 expression is specific to virus infection with this gene and whole T cells are more sensitive than T regs to the up-regulation of Klf4 gene after infection. This can be explained by the role of Klf4 on T cell activation and differentiation. Klf4 acts on the IL7a gene driving the differentiation of Th17 T cell subset. Thus, on the heterogeneous population of peripheral blood T cells there are going to be naïve T cells or other helper T cells that are more sensitive responding to exogenous Klf4 and viral infection as a whole, whereas T regs are already a committed T cell subset explaining the less responsive effect on Klf4. On the other hand, the expression of Oct4, Sox2 and c-Myc was comparable to that of T cells. Also, T regs infected with lentivirus had similar expression patterns than those infected with retrovirus (Figure 15).

In a different experiment, re-stimulated umbilical cord blood (UCB) T regs were transduced with lentivirus and 2 different concentrations of doxycycline were tested (1

and 2 μg/mL). As doxycycline controls the cargo gene expression we tested if whether or not a higher concentration would help increase the expression levels of the 4 transgenes (Qin *et al.,* 2010; Vogel *et al.,* 2004). Expression analysis of OSKM transcripts by qRT-PCR showed no significant difference between these two groups or the control, suggesting that either the viral construct or doxycycline dose was not optimal for transgene expression in T regs. Also, Klf4 levels were not detectable on UCB T regs compared to peripheral blood T regs (Figure 16).

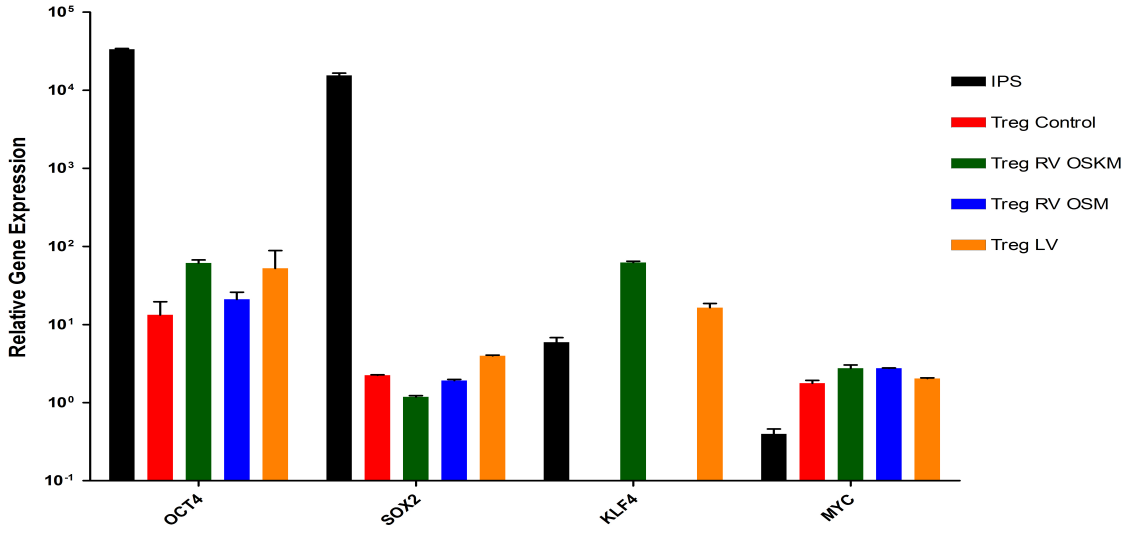


Figure 15. Gene expression analysis in peripheral blood regulatory T cells. Quantitative RT-PCR analysis of Oct4, Sox2, Klf4, and cMyc transcript expression levels in T regs infected with four individual retroviral vectors containing Oct4, Sox2, Klf4 and cMyc (Treg RV OSKM). T regs infected with three different retroviral vectors containing Oct4, Sox2, and cMyc (T reg RV OSM). T regs infected with Lentivirus and treated with 1 μg/mL of doxycycline (Treg LV). Gene expression is relative to untreated T regs (Treg control). All values are normalized against endogenous GAPDH expression. IPS, induced pluripotent stem cells; Treg, regulatory T cells; RV, retroviral vector; LV, lentiviral vector; OSKM, Oct4, Sox2, Klf4, cMyc.

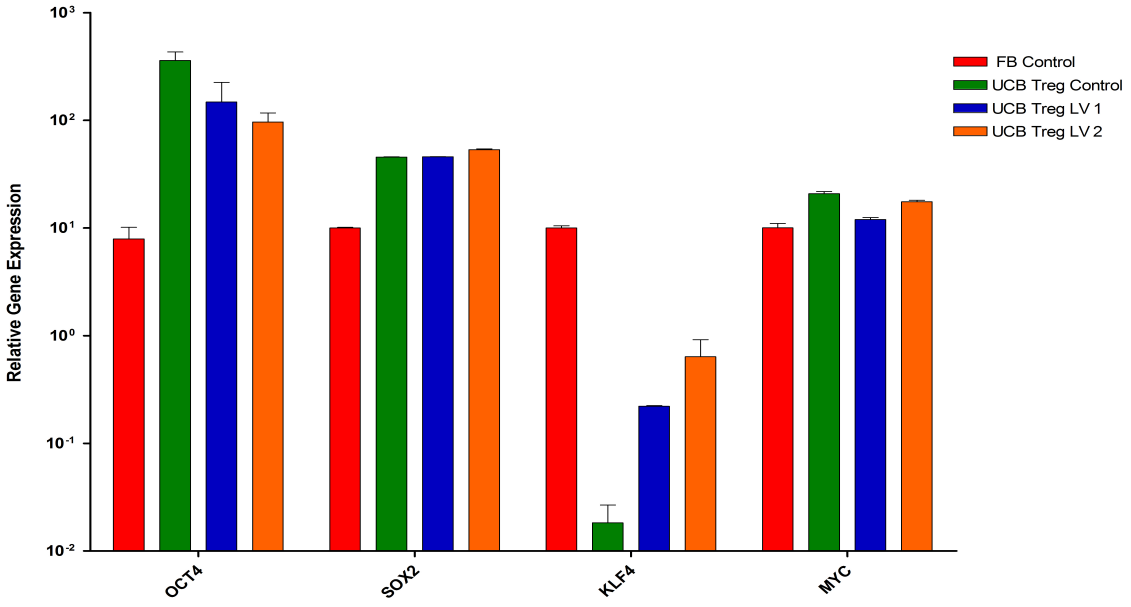


Figure 16. Gene expression analysis in UCB regulatory T cells. Quantitative RT-PCR for Oct4, Sox2, Klf4, and cMyc transcript levels of UCB T regs infected with lentivirus 48 hours post doxycycline treatment. Infected cells were treated with two different concentrations of doxycycline: 1 and 2 μg/mL (UCB Treg LV 1 and UCB Treg LV2 respectively). UCB Treg without LV infection (UCB Treg control). Gene expression is relative to normal human fibroblast control (FB control). All values are normalized against endogenous GAPDH expression. FB, fibroblasts; UCB, umbilical cord blood; Treg, regulatory T cells; LV, lentiviral vector.

2. Reprogramming with Sendai virus vector

Peripheral blood T regs were re-stimulated and infected with Sendai virus at an MOI of 5. At day 25-post infection, 4 iPSC-like colonies were seen (see representative example in Figure 17). Potential colonies were manually picked and reseeded on fresh seeded iMEF plates. After 3 passages colonies no longer grew, suggesting that the colonies were partially reprogrammed cells. We believe that a higher MOI will increase the efficiency of T reg-iPSC formation, however this remains to be confirmed. Even though we did not see fully reprogrammed colonies with this approach, the Sendai virus seems to be a better approach to derive iPSC from T cell subsets when compared to the retrovirus and lentivirus. Experiments are ongoing to generate Treg-iPSC with a higher MOI.

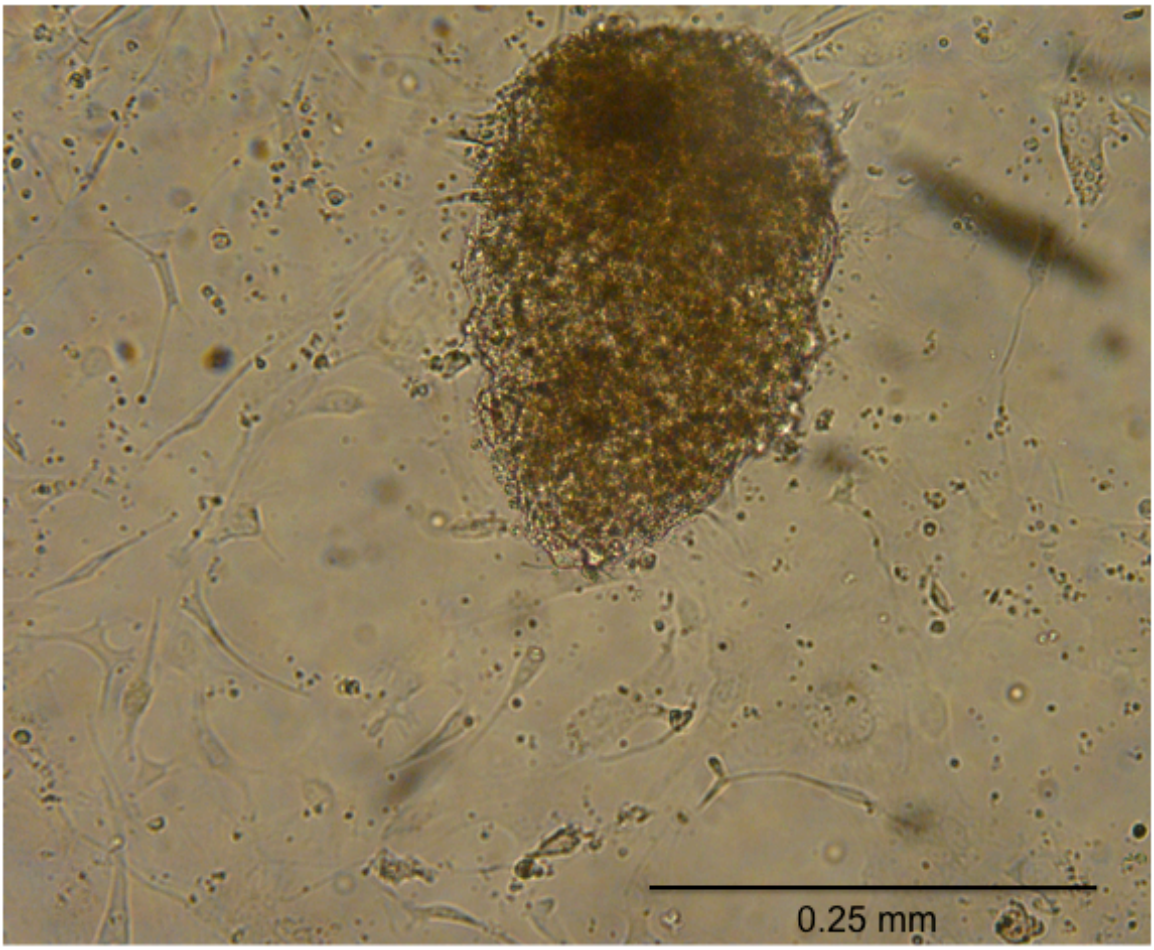


Figure 17. iPSC-like colony derived from T regs infected with Sendai virus. Image of iPSC-like colony at day 25 post Sendai virus infection.

Retrovirus optimization experiments

Our retrovirus construct has been successful in deriving iPSC from fibroblasts, indeed, being the goal standard for deriving fibroblasts-iPSC in our lab. However, this was not the case with T cells. As part of this project we aimed to optimize the retrovirus construct to enhance viral infection and subsequently Tcell-iPSC formation. Previously we analyzed retrovirus infection by measuring GFP expression on flow cytometry. All four retrovirus contain GFP gene, thus obstructing the analysis of each of the transgenes individually. With this in mind, we first wanted to test if all four retrovirus were able to infect the T cells, we did this by infecting T cells and T regs with all four retrovirus together and individually with unconcentrated virus (as previously used). We also tested infecting the cells with all four retrovirus concentrated to increase the MOI during infection. In addition we compared the expression levels when using fresh versus frozen virus. We studied the OSKM gene expression on T cells and T regs after infection by qRT-PCR and GFP expression by flow cytometry.

1. T cells

Seven experimental conditions (cells infected with Oct4 only, Sox2 only, Klf4 only, cMyc only, all four factors, 100 μL of concentrated virus and 500 μL of concentrated virus) and one control (T cells without infection) were used with fresh virus and the same 8 groups were used with frozen virus. Figures 18 and 19 show the flow cytometry data for GFP and gene expression using qRT-PCR in T cells infected with frozen or fresh virus.

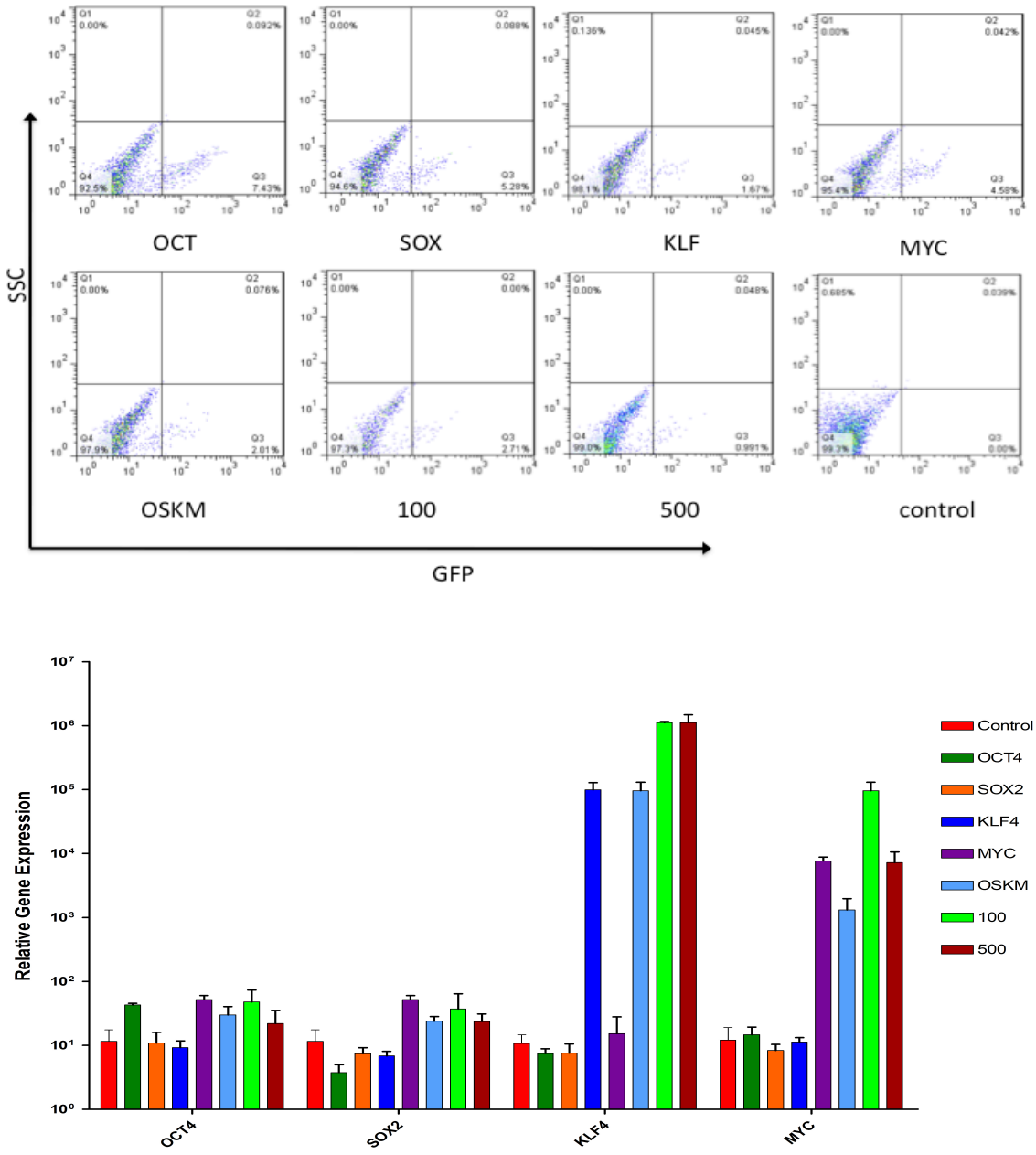


Figure 18. Peripheral blood T cells infected with fresh Retrovirus. Top figure shows flow cytometry analysis of GFP expression on peripheral blood T cells infected with fresh un-concentrated retrovirus containing either Oct4, Sox2, Klf4, or cMyc individually or with all 4 retroviruses in a 1:1:1:1 ratio (OSKM). T cells were also infected with two different volumes of all 4 retroviral vectors concentrated on a 1:1:1:1 ratio as well (100, 100 µL; 500, 500 µL). Bottom figure represents quantitative RT-PCR analysis for Oct4, Sox2, Klf4, and cMyc transcript expression on each of the experimental groups described. Gene expression levels are relative to untreated T cells (control). All values are normalized to endogenous GAPDH expression.

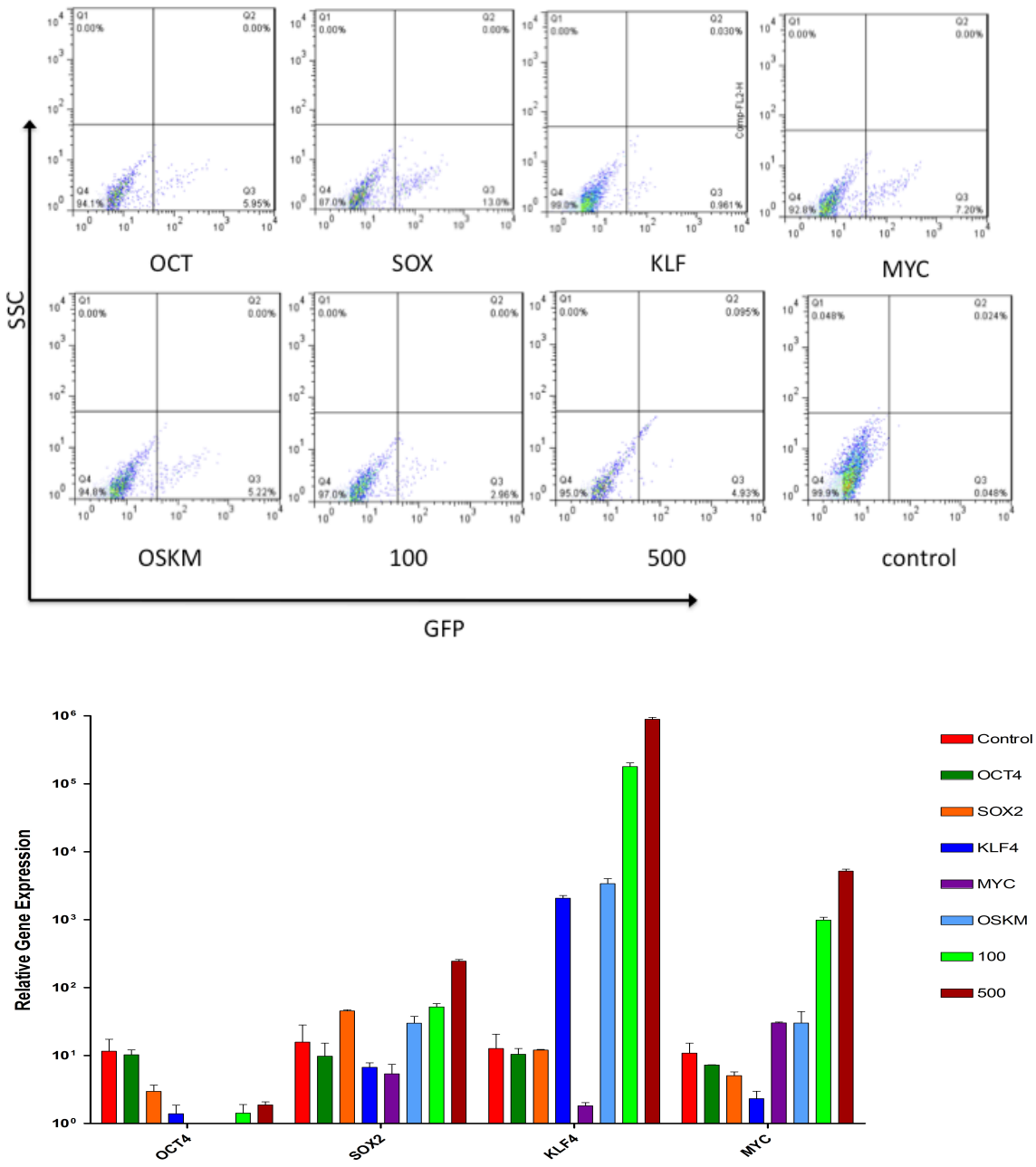
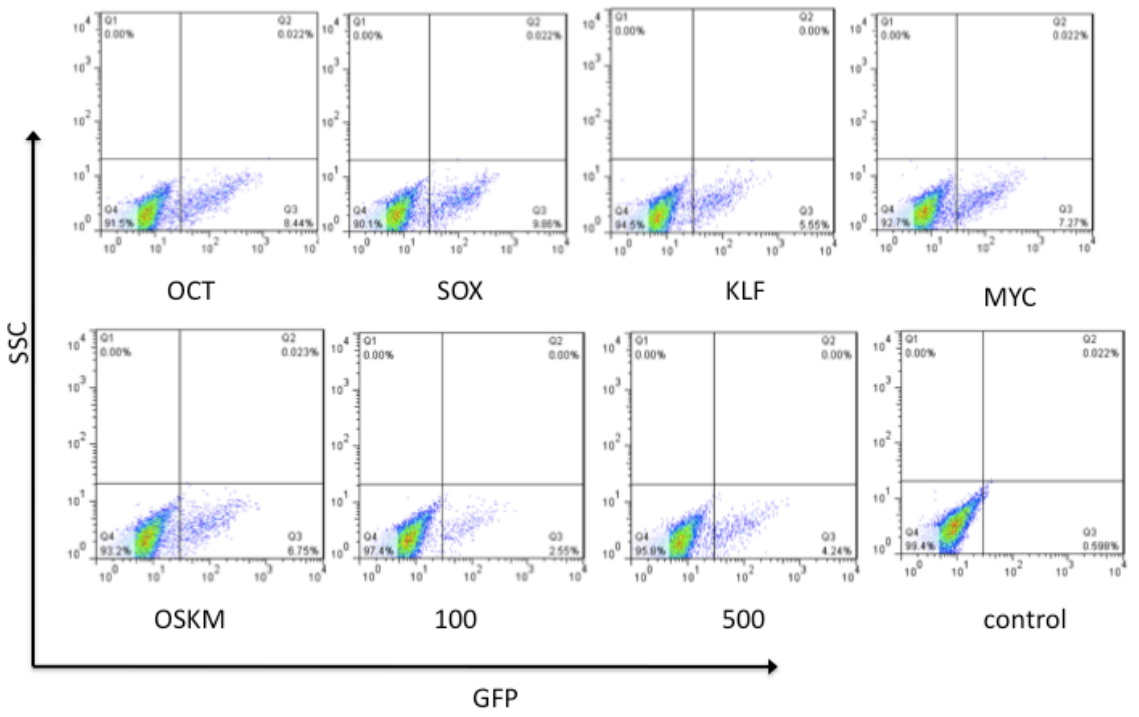


Figure 19. Peripheral blood T cells infected with frozen Retrovirus. Top figure shows flow cytometry analysis of GFP expression on peripheral blood T cells infected with frozen un-concentrated retrovirus containing either Oct4, Sox2, Klf4, or cMyc individually or with all 4 retroviruses in a 1:1:1:1 ratio (OSKM). T cells were also infected with two different volumes of all 4 retrovirus concentrated on a 1:1:1:1 ratio as well (100, 100 µL; 500, 500 µL). Bottom figure represents quantitative RT-PCR analysis for Oct4, Sox2, Klf4, and cMyc transcript expression on each of the experimental groups described. Gene expression levels are relative to untreated T cells (control). All values are normalized to endogenous GAPDH expression.

2. T regulatory cells

The retroviral vectors were also examined with T regs. Seven experimental conditions (cells infected with Oct4 only, Sox2 only, Klf4 only, cMyc only, all four factors, 100 μL of concentrated virus and 500 μL of concentrated virus) and one control (T cells without infection) were used with fresh virus and the same 8 groups were used with frozen virus. GFP expression and transgene expression were analyzed with flow cytometry and qRT-PCR respectively (Figure 20 and 21).

There is a dichotomy between the GFP and the OSKM gene expression, in which GFP expression is seen but no OSKM expression is evident or vise versa. Our first hypothesis was that the viral construct was not working properly, thus we investigated this issue with human fibroblasts that we knew could be successfully infected with retrovirus. We infected normal neonatal human fibroblasts with Oct4, Sox2, Klf4 and cMyc retrovirus individually and 48 hours post second infection the cells were sorted for GFP positive and negative. We then isolated total RNA from these two groups (GFP positive and GFP negative) and qRT- PCR was done for OSKM gene expression (Figure 22). Fibroblasts that were GFP positive after infection with Klf4 and cMyc showed elevated gene expression compared to GFP negative fibroblast infected with the same virus. However, fibroblasts infected with Oct4 or Sox2 that were GFP positive did not show up-regulation of Oct or Sox transcripts.



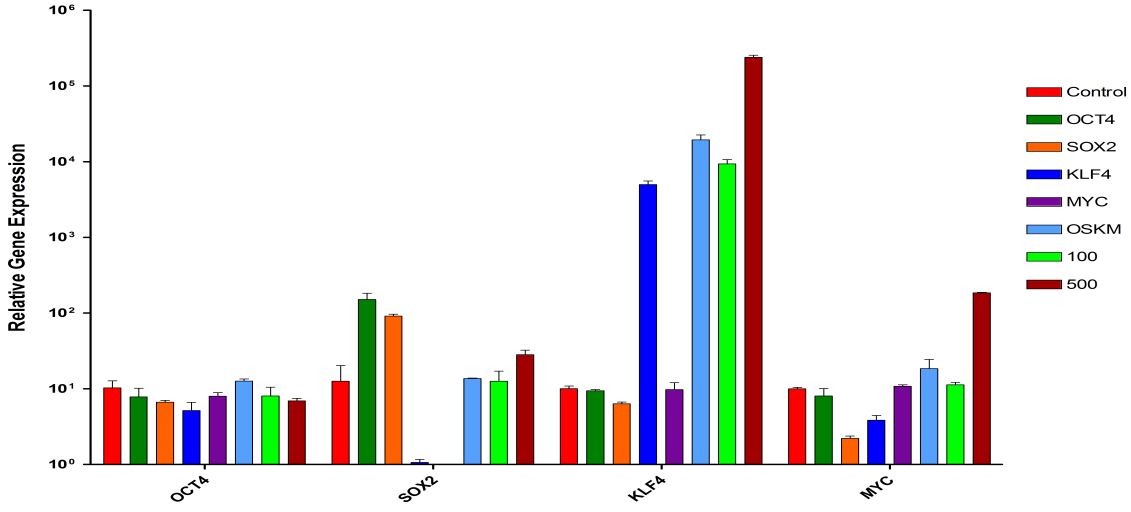


Figure 20. Peripheral blood regulatory T cells infected with fresh Retrovirus. Top figure shows flow cytometry analysis of GFP expression on T regs infected with fresh un-concentrated retrovirus containing either Oct4, Sox2, Klf4, or cMyc individually or with all 4 retroviruses in a 1:1:1:1 ratio (OSKM). T cells were also infected with two different volumes of all 4 retrovirus concentrated on a 1:1:1:1 ratio as well (100, 100 µL; 500, 500 µL). Bottom figure represents quantitative RT-PCR analysis for Oct4, Sox2, Klf4, and cMyc transcript expression on each of the experimental groups described. Gene expression levels are relative to untreated T cells (control). All values are normalized to endogenous GAPDH expression.

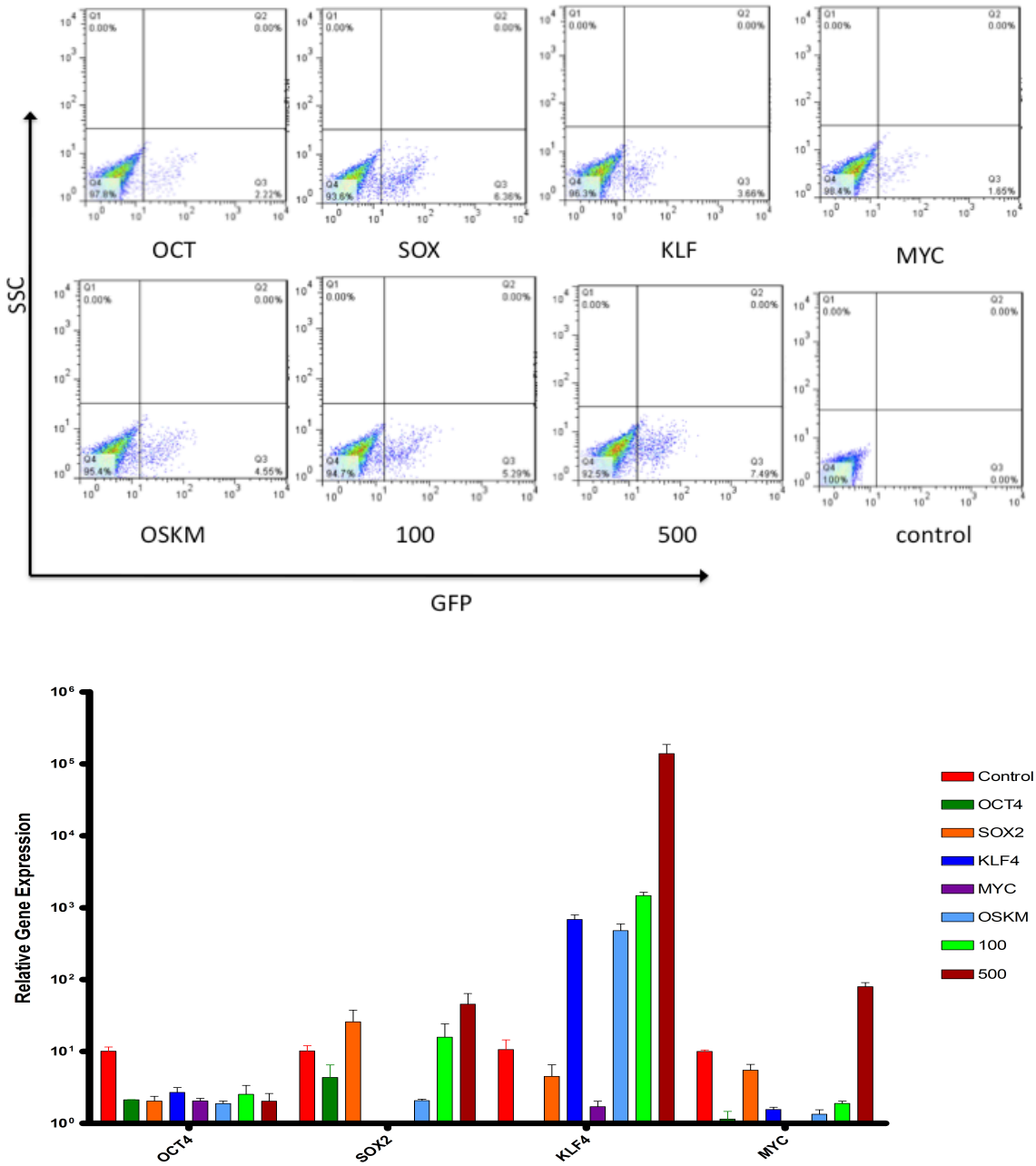


Figure 21. Peripheral blood regulatory T cells infected with frozen Retrovirus. Top figure shows flow cytometry analysis of GFP expression on T regs infected with frozen un-concentrated retrovirus containing either Oct4, Sox2, Klf4, or cMyc individually or with all 4 retroviruses in a 1:1:1:1 ratio (OSKM). T cells were also infected with two different volumes of all 4 retrovirus concentrated on a 1:1:1:1 ratio as well (100, 100 μL; 500, 500 μL). Bottom figure represents quantitative RT-PCR analysis for Oct4, Sox2, Klf4, and cMyc transcript expression on each of the experimental groups described. Gene expression levels are relative to untreated T cells (control). All values are normalized to endogenous GAPDH expression.

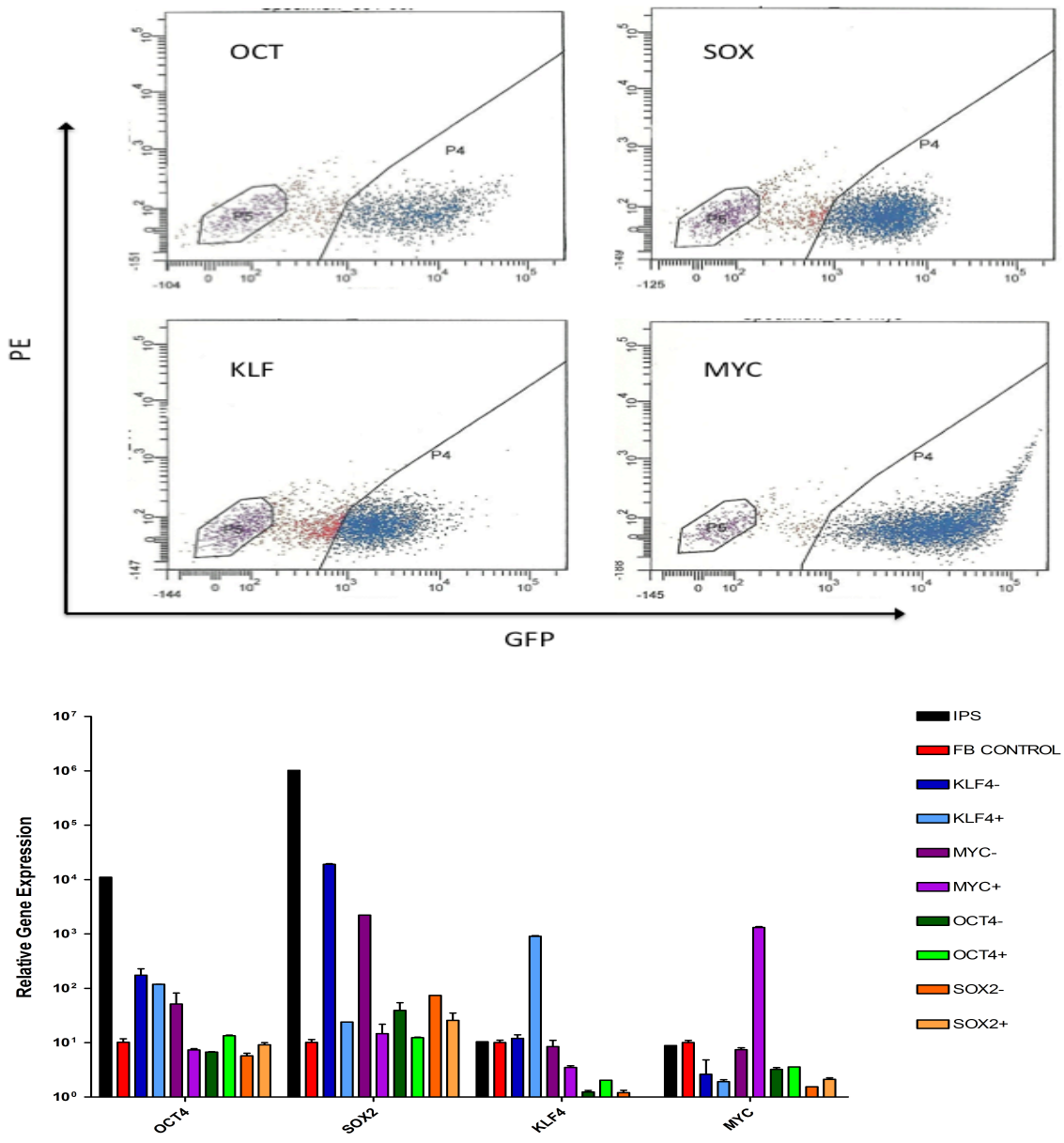


Figure 22. Human fibroblasts infected with Retrovirus. Human fibroblasts (hFB) were infected with each retrovirus individually (Oct4, Sox2, Klf4, and cMyc) and sorted by flow cytometry for GFP negative and positive populations on each experimental group. Top figure shows the GFP negative (P5) and GFP positive (P4) on hFB infected with retrovirus containing Oct4, Sox2, Klf4, or cMyc individually. Sorted cells (GFP positive (+) and negative (-)) were analyzed for Oct4, Sox2, Klf4, and cMyc transcript levels by qRT-PCR (bottom figure). Gene expression levels are relative to untreated human fibroblasts (FB control). All values are normalized to endogenous GAPDH expression. IPS, induced pluripotent stem cells; FB, fibroblasts; +, GFP positive cells; -, GFP negative cells.

Reprogramming central memory and effector memory T cells to iPSC

1. Sendai virus vector

In our studies with peripheral blood T cells we showed that the optimal platform for T cell reprogramming is the Sendai virus at an MOI of 20. Thus, we investigated if the same platform was able to reprogram memory T cells to iPSC. Memory cells were kindly provided by Mark Jensen Laboratory (University of Washington, Seattle, WA). Experiments are in progress with both effector and central memory T cells infected with Sendai virus at an MOI of 20.

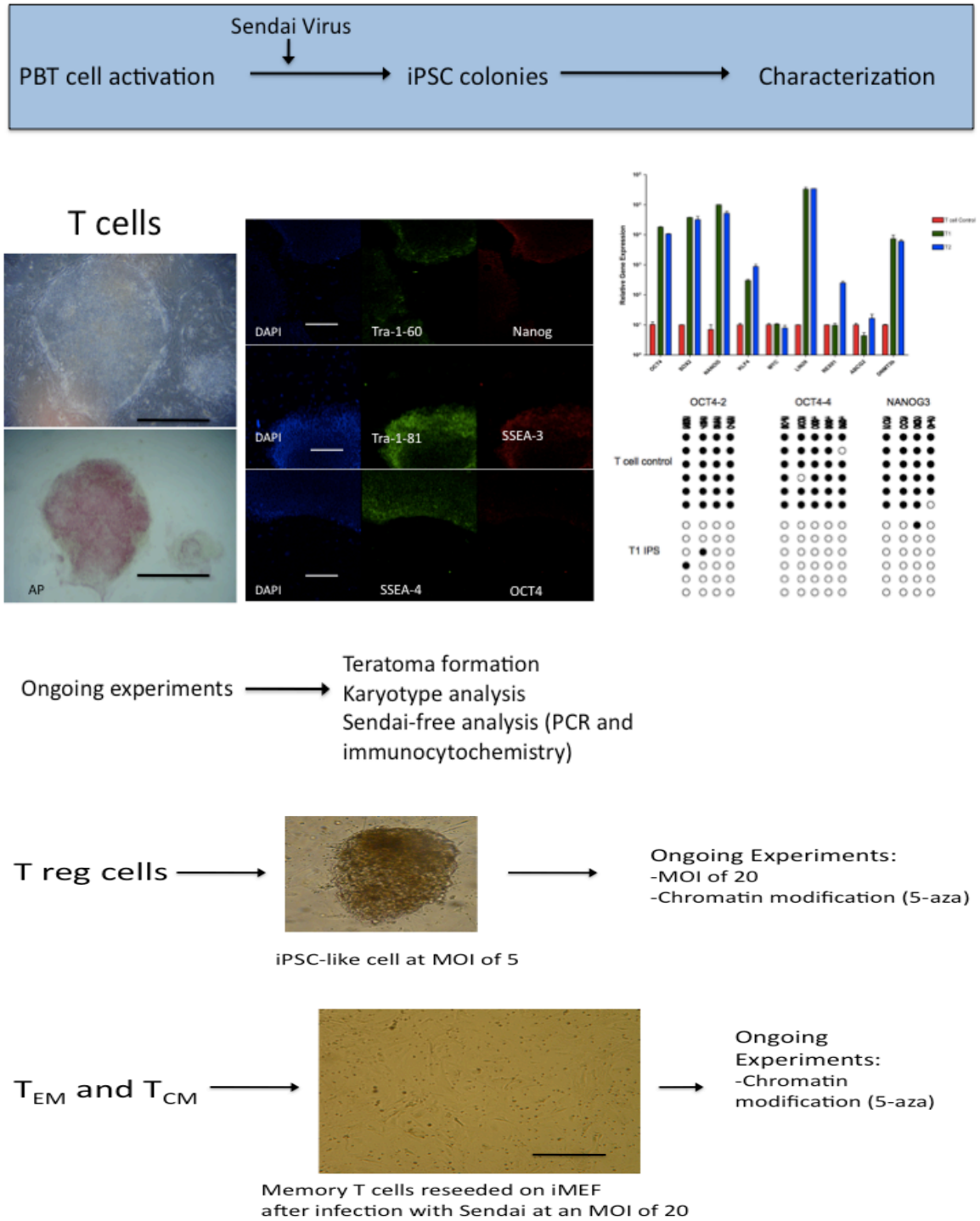


Figure 23. Summary scheme of results. Summary of results from Sendai virus experiments with peripheral blood T cells, peripheral blood regulatory T cells, and effector memory and central memory T cells. PBT, peripheral blood T cells; MOI, multiplicity of infection; 5-aza, 5-aza-2-deoxycytidine; iPSC, induced pluripotent stem cells; AP, alkaline phosphatase. Black and white bar scales are 0.50 mm and 50 μm respectively.

DISCUSSION

Our goal was to investigate the requirements for reprogramming various T cell types with the ultimate goal of developing the optimal method to produce iPSC from effector and central memory T cells. Given that whole T cells have been shown to reprogram successfully with Retrovirus (Brown *et al*., 2011) and Sendai virus (Seki *et al*., 2010; Okano *et al.,* 2003) we began testing whole T cells with different vectors to establish the optimal platform on a non-specific population of T cells before testing on specific T cell subsets.

Our data demonstrate that the optimal platform for T cell reprogramming is the Sendai virus vector at an MOI of 20. We show that a higher MOI correlates with efficiency of iPSC formation, confirming previous studies (Seki *et al*., 2010). In addition, our data show that Tcell-iPSC derived with Sendai virus show methylation and gene expression pattern, pluripotency markers, and alkaline phosphatase activity, all correlating with characteristics of a pluripotent cell (iPSC and ESC). However, experiments are ongoing to characterize the resultant iPSC in their ability to form teratomas on immunocompromised mice, which is a key characteristic of pluripotent cells. In addition, we are testing if the iPSC are free of Sendai virus vector, to assess this we plan on performing qRT-PCR with primers specific to transgenes in addition to immunostaining with anti-SeV. Since the iPSC were derived from a normal donor and its origin was confirmed by HLA typing on both donor and iPSC, we are also performing a karyotype analysis to examine chromosomal abnormalities that could result from the reprogramming process and *in vitro* maintenance. Analyzing the genomic integrity of iPSC is of great importance to assure the safety of iPSC if regenerative therapies are to be conducted in the future (Lund *et al.,* 2012).

When testing the Sendai virus with T regs at an MOI of 5 we were able to detect iPSC-like colonies. Although, these iPSC were not able to grow at later passage, suggesting that these colonies were partially reprogrammed, we believe that a higher MOI will enable the derivation of Treg-iPSC as we saw with whole T cells. On the other hand, this

platform is to be optimized for reprogramming T_{CM} and T_{EM}. It is known that reprogramming mature cells is more challenging than reprogramming an immature cell (Gurdon and Wilmut, 2011). Mostly because of their methylation patterns and chromatin modifications that prevents robust gene expression of earlier phase genes (e.g. pluripotency genes); critical for successful reprogramming. To this end, other groups have shown that chromatin modification treatment before and during the reprogramming process positively influence the development of iPSC. Ascorbic acid treatment was shown to attenuate hypermethylation of maternally imprinted genes, commonly silenced in iPSC, in mature B cells (Stadtfeld *et al.,* 2012). Another study with B cells showed that treatment with 5-Aza-2'-Deoxycytidine (5aza), a methyltransferase inhibitor, also attenuated hypermethylation of maternal imprinted genes resulting in increased efficiency of iPSC formation and iPSC with high developmental potential (Wesemann *et al.,* 2012). We are currently optimizing the memory T cell reprogramming experiment by incorporating the 5aza treatment before and during reprogramming with Sendai virus. We hypothesize that this treatment will support the formation of iPSC from memory T cells by preventing hypermethylation of key promoters for pluripotency, like Nanog and Oct promoters. To analyze this, we plan on evaluating the methylation pattern on these promoters with bisulfite sequence analysis before and after treatment. It is important to note that though iPSC from a specific T cell subset have not been reported to date, Tcell-iPSC have been reported from T cell lymphocytes on periphery. T cells in periphery are mostly naïve T cells, which are in the earliest phase on their mature life. T_{CM} and T_{EM} are the most mature form a T cell can achieve and they are less frequent than naïve T cells on periphery. Thus, it is possible that Tcell-iPSC derived on previous reports and in our studies were derived from naïve T cells rather than effector or memory T cells. Furthermore, T_{CM} and T_{EM} are prone to apoptosis when stimulated correlating to their decrease telomere length (Sallusto et al., 2002), this and the fact that memory cells are the most mature form of a T cells could be additional reasons these cells are more difficult to reprogram to a pluripotent state.

Our retrovirus and lentivirus experiments show that Klf4 transcript levels were upregulated upon viral transfection; nonetheless this was not the case for the Oct4 and Sox2 transcript levels. Oct4 and Sox2 expression is critical for the development of iPSC, thus suggesting an explanation why these two vectors were unable to reprogram T cells. The qRT-PCR data in our retrovirus optimization experiment with human fibroblast show that the Klf4 and cMyc retrovirus were able to up-regulate these transcripts on fibroblast but the virus containing Oct4 and Sox2 were unable to do so. The human fibroblast data correlates with our previous qRT-PCR data analysis on T cells. Even though our data shows that OSKM transcripts levels were similar when infecting T cells with lentivirus or retrovirus, the fact that our retrovirus construct consists of four different viruses each with a different pluripotency gene makes it more of a challenge to obtain cells with all four transgenes, decreasing the possibility of obtaining iPSC with this construct. Additional experiments need to be done to optimize integrating vectors for T cell reprogramming. Important measures to consider are the activation status of T cells during transfection, optimal doxycycline dose during reprogramming and chromatin manipulations.

The field of induced pluripotency and lineage conversion is an ongoing area of research and many questions need to be answered to completely understand pluripotency. For example, how important is the role of chromatin modifications in the process of reprogramming? Why does only a minority of cells respond to cellular reprogramming? How does factor-induced transdifferentiation differ from iPSC reprogramming? (Graf, 2011). Blood cells have been shown over the years to be plastic in changing their cell fate. Kulessa and his group (Kulessa *et al*., 1995) forced the expression of the transcription factor GATA-1, expressed in erythroid, thrombocytic and eosinophilic derivatives, on myeloblasts that resulted in suppression of myelomonocytic markers and reprogramming into eosinophils or thromboblasts. More recently Laiosa *et al*. (2006) showed that fully committed pre-T cells were inducible towards the formation of functional macrophages by the expression of transcription factor C/EBP alpha. The last two experiments show the plasticity of blood cells in redirecting cell fate by expression of

lineage specific transcription factors, the same plasticity is seen with repression of transcription factors (Rhodes *et al*., 2005) driving differentiation of committed cells to a different lineage within the hematopoietic system. The fact that the hematopoietic system has many ramifications of cell lineages makes it ideal to study cell fate and conduct lineage conversion experiments. Generating iPSC from different T cell subsets, including memory cells can contribute to answering these questions. Epigenetic studies before and after reprogramming will permit the understanding of the importance of chromatin modifications.

The ability to generate Tcell-iPSC provides the opportunity to study T cells on a different level. T cell development, epigenetics, immunological disease modeling, drug screening and T cell therapy are some of the applications for the use of Tcell-iPSC. Moreover, the production of iPSC from memory T cells will enable epigenetic studies that could help understand in more depth the key differences between central and effector memory cells. Developmental studies using memory T cell iPSC could also help understand how memory cells develop and become either effector or central memory. Furthermore, in theory, iPSC derived from memory cells will sustain the epigenetic memory of a T cell, including the TCR rearrangement. This is of great interest for cell therapy because it will enhance the differentiation of T cell-iPSC to a T cell subset of interest for the treatment of disease. The data shown in this manuscript may therefore be of great importance for the development of iPSC from T cell subsets without genomic integration for cell therapy and molecular studies. Furthermore, it provides an insight to acquiring pluripotency in cell types of varying differentiation state and a potential formula design for epigenetic manipulations.

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