GENERATION OF INDUCED PLURIPOTENT STEM CELLS AND MESENCHYMAL STROMAL CELLS

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Abstract

Mesenchymal stromal cells (MSCs) are a population of mesoderm-derived cells that possess the ability to differentiate into bone, cartilage, and adipose tissue. They are important to understanding the developmental process of musculoskeletal tissue, and can be utilized for novel human cell therapies. Previous studies by our group and others have demonstrated development of MSCs from human embryonic stem cells (hESCs). Now, we have identified a population of potential mesenchymal precursor cells from adult bone marrow derived iPSC lines using CD73 as a selection marker. Sorting and culture of the hESC/iPSC-derived CD73-positive cells lead to development of MSCs capable of making bone, cartilage, and adipocytes. However the variations in differentiation methods have been found to strongly influence their mesenchyme induction and their ability to make bone, cartilage and adipose tissue. We compare the spin EB (embryoid body) versus stromal co-culture techniques to arrive at a MSC population in our studies. Additionally, these studies examined novel ways to derive iPSCs that could be used for derivation of MSCs and other cell populations. Induced pluripotent stem cells have previously been generated from human dermal fibroblast cells. However, the requirement for skin biopsies and the need to expand fibroblast cells for several passages in vitro make it a cumbersome source for generating patient-specific stem cells. Reprogramming from human blood cells represents a consistent method of establishing patient-specific iPSCs.

Table of Contents

List of figures……iv
Introduction ……..…….1
Materials and Methods …… 6
Results ……...13
Discussion ……20
Figure Captions ……22
Figures ……...23
References ……..…..29

List of Figures

Figure 1……23

Figure 2……24

Figure 3……25

Figure 4……26

Figure 5……27

Figure 6……28

Introduction:

Characterization of Mesenchymal Stromal cells:

In 1998, by harvesting the inner cell mass of pre-implantation fertilized embryos and subsequently culturing these cells on a mouse embryonic feeder layer, the first human embryonic stem cell line was passaged [Thomson et al., 1998]. Typically, these hESCs can remain in an undifferentiated pluripotent state indefinitely while still possessing the ability to undergo differentiation into the three germ layers— endoderm, ectoderm, and mesoderm—that ultimately give rise to every cell in the adult organism.

These cells possess great potential for cell-replacement therapies for a variety of congenital diseases, injuries, treatment of cancers, and other disorders. The hESCs also serve as ideal models for drug screening and toxicology studies. From human embryonic stem cells, functional neurons [Reubinoff et al., 2001] [Zhang et al., 2001], cardiac muscle [Kehat et al., 2001], hematopoietic cells [Kaufman et al., 2001], endothelial cells [Levenberg et al., 2002] and a variety of other cell types have been differentiated.

Mesenchymal stem/stromal cells (MSCs) are defined as the mesoderm derived cells that can differentiate into bone, cartilage and fat. Bone marrow, adipose tissue, muscle, umbilical cord blood and other connective tissues have been used to isolate MSCs while these have shown to be derived from human embryonic stem cells as well as induced pluripotent stem cells [Lazarus et al., 1995] [Guilak et al., 2004] [Young et al., 2001] [Seo et al., 2004] [Erices et al., 2000] [Caplan et al., 2007]. These cells can be characterized by the expression of certain standard cell surface markers including CD73, CD90, CD105, CD44 and CD146 while lacking the typical hematopoietic markers, their high proliferation rate and their ability to adhere to plastic surfaces.

Owing to their ability to differentiate into osteogenic precursors, MSCs have the potential to be used in tissue engineering for bone repair and regeneration of damaged tissue [Petitie et al., 2000] [Uematsu et al., 2005]. They could possibly be used in therapies to repair infarcted myocardium [Shake et al., 2002] [Zhang et al., 2005, 2006]. Despite the enormous clinical potential of MSCs, adult MSCs are often isolated as a heterogeneous population of cells with a variation in the trilineage differentiation kinetics amongst

various tissue populations. Currently, no cell therapy that uses adult stem cells for bone repair or regeneration has yet found way into a clinical trial.

Thus, hESC or IPSC derived MSC could well be the solution to the above bottlenecks in regenerative medicine. However, it is important to understand the developmental mechanics of generation these hESC/iPSC derived MSCs. Previously, our and other groups have used a stromal cell co-culture method to support mesenchymal induction from the pluripotent cells. Previous studies have also been able to isolate MSCs from hESC differentiation cultures. Barberi et al. isolated $CD73^+$ adherent fibroblast-like cells from hESCs cocultured with OP9 cells that were phenotypically and functionally similar to bone marrow MSCs. Trivedi and Hematti isolated $CD73^+$ cells in OP9 co-culture that again were phenotypically and functionally similar to MSCs; however, these cells were isolated in conjunction with functional hematopoietic progenitor $CD34^+$ cells [Trivedi et al., 2007]. Our group advanced the above studies with the demonstration that hESC-derived $CD34^+$ $CD73^-$ cells co-cultured on a stromal layer can serve as MSC progenitor cells with the ability to differentiate into adipocytes, osteoblasts and chondrocytes [Kopher et al., 2010].

As mentioned briefly, another differentiation technique of hESCs apart from co-culture of hESC with a stromal layer is the embryoid body method - mechanically or enzymatically digested clumps of undifferentiated ES cells stimulate the formation of embryoid bodies. However this technique is subject to variability, as the quality of the starting hESC population, the size of the hESC clumps that initiate EB formation, the presence of feeder cells and the cell-associated or secreted factors associated with stromal cell cocultivation influence the results of the differentiation [Ng et al., 2008]. Elefanty's group introduced the "spin EB" method of differentiation that overcomes the above mentioned shortcomings. This method used a known number of undifferentiated cells that are aggregated in each well of a 96 well round-bottomed low-attachment plate, resulting in the reproducible formation of EBs of uniform size in each well. A defined media- BPEL (Bovine Serum Albumin (BSA) Polyvinylalchohol Essential Lipids) was used for differentiation of EBs. The spin EB method is now effectively used for the hematopoietic differentiation of hESC and iPSCs.

In these current studies, we have chosen to compare the efficiency of a stromal-free differentiation (spin EB) method versus the stromal-based co-culture method for generating MSCs and subsequently, osteogenic progenitor cells.

Generation of Induced pluripotent cells from peripheral blood mono nuclear cells:

Yamanaka first showed that the exogenous expression of four genes (Oct4, Sox2, Klf4, and c-Myc) in somatic cells led to the generation of self-renewing cell lines that had the potential to differentiate into multiple lineages [Takashi et al., 2007]. These cells were defined as IPSCs and they were identified to be functional counterparts of hESCs. Human iPSCs have been shown to form teratomas with contributions of all three germ layers (endoderm, ectoderm, and mesoderm) and have been utilized to produce many differentiated cell lineages [Takashi et al., 2007]. Subsequently, Thomson and colleagues found that Oct4, Sox2, Nanog, and Lin28 are sufficient for the generation of human iPSCs and they achieved this using a lentiviral vector [Yu et al., 2007]. However these methods were far from clinical translation, as there remain questions about the efficiency of generation of iPSCs, genomic integration of the transcription factors in the target cells, and the risk of tumors as most of the reprogramming factors are oncogenic in nature. Currently, iPSCs can be effectively generated by transient gene expression of one or two reprogramming factors rather than requiring stable genome integration [Yu et al., 2009].

Overall, this technology has brought us a step closer to the concept of generation of patient-specific iPSCs, where recombinant methods within their iPSC line could hypothetically reverse the disorder and then this “treated” line is differentiated into the original tissue type for replacement.

The clinical translation of the iPSC technology is deemed ambitious by many. However, methods utilizing lentiviral integrations, excisable transposons, non-integrating plasmids, non-integrating viral vectors, micro RNAs and protein transduction of reprogramming factors are being established [Kaji, Norrby et al. 2009] [Yu et al. 2009]. Small molecules that directly activate the expression of reprogramming factors from the somatic genome have been reported to produce pluripotent stem cells. [Ichida, Blanchard et al. 2009] Development of a standard high efficiency method to derive iPSC lines without genomic

integration, stringent tests for pluripotency and improvement of screening criteria for true iPSC lines could make this a reality.

In collaboration with a group from Mayo clinic, we aimed to generate patient specific human iPSCs from autologous blood cells for utilization in cell therapy for cardiovascular disease. iPSC technology from autologous blood cells blood MNCs offers several advantages over other cell types [Chou et al, 2011]. It is more convenient (in terms of culture condition and duration) and less invasive to obtain peripheral blood (PB) than dermal fibroblasts and keratinocytes. In addition, using peripheral blood allows access to numerous frozen samples already stored at blood banks. Such samples could be expanded in culture and reprogrammed to iPSCs, which in turn allows researchers to study the molecular mechanism underlying blood and other disorders [Jaenisch et al., 2010]. The ability to reprogram cells from human blood will allow the generation of patient-specific stem cells for diseases in which the disease-causing somatic mutations are restricted to cells of the hematopoietic lineage [Daley et al., 2011].

Mostoslavsky's group generated a Stem Cell Cassette (STEMCCA) containing all four factors (Oct4, Klf4, c-Myc, Sox2) within a single lentiviral vector using a combination of 2A peptide and internal ribosome entry site (IRES) technology (Sommer et al., 2009). This lentivirus produces a single multicistronic transcript with Oct4, Klf4, Sox2 and c-Myc expressed under a single elongation factor promoter, to ensure ubiquitous and stable expression. This vector allows for the generation of four separate and functional factors driven by the same promoter. Also, the Cre-loxP system can be used to excise the cassette, since it is flanked by loxP sites after integration into the genome (Sommer et al., 2010). In our first attempt to make iPSCs from patient blood samples, we used STEMCCA vector in order to deliver the OCT4, KLF4, SOX2, and c-MYC transcription factors to the PBMCs. This method was selected due to the high efficiency of reprogramming while still maintaining the potential for excision.

Subsequently, a method of iPSC derivation without altering the genome was preferred, ideally by a virus-free approach. Recently, several virus-free and integration-free methods were reported, which generated mouse and human iPSCs by using purified proteins or mRNAs, as well as plasmids. Two of these studies used plasmid-based

episomal vectors to successfully reprogram human fetal neural progenitor cells and neonatal fibroblasts, generating integration-free iPSC lines. Inclusion of the EBNA1 gene and the OriP DNA sequence from the Epstein-Barr virus (EBV) enables extra-chromosomal replication. However, although a combination of three EBNA1/ OriP expressing seven genes were used in DNA transfection the efficiency of reprogramming was very low [Yu et al., 2009]. It is therefore essential that development of more efficient methods to generate integration- free and virus-free human iPSCs from adult somatic cells be given due importance.

Linzao Cheng's group in 2011 [Chou et al., 2011] reported increased efficiency of reprogramming cord blood cells using two episomal vectors – the plasmid containing the reprogramming genes along with plasmid that encoded the SV 40 T-antigen. This was of considerate interest to our group, and we obtained the vectors to attempt reprogramming of PBMCs to iPSCs.

Materials and Methods:

Cell culture:

Culture of human embryonic stem cells (hESC): Human embryonic stem (H9 cell lines, University of Wisconsin) cells were cultured on irradiated mouse embryonic fibroblasts (MEFs) (Chemicon). MEFs were plated onto gelatin-coated NuncΔ plastic tissue culture plates (Thermo Scientific) at a density of 200,000 cells per well of a six well plate. The cells were provided hESC media which consisted of Dulbecco's modified Eagle medium-F/12 (DMEM-F/12) supplemented with 15% Knock-Out Serum Replacer (KOSR), 2mM L-glutamine, 0.1mM β-mercapto ethanol (β –ME), 1% non-essential amino acids (NEAA), penicillin/streptomycin (P/S) (all from Invitrogen), and 8 ng/mL basic fibroblast growth factor (R&D Systems). hESCs were passed by Collagenase IV (1mg/mL) treatment and mechanical dissociation using a glass pipette. The collagenase in the cell suspension is diluted by addition of hESC media and this suspension is centrifuged at 1300 rpm for 3.15 minutes and the cell pellet is resuspended in appropriate amounts of fresh ES media and distributed over fresh feeder layer.

Setting up hESC derived spin EBs: Spin EBs were set up from H9s at passage 64, and enzymatically (TripLE, GIBCO)-adapted for 24 passages as described in the protocol by Elefanty's group in 2008 [Ng et al., 2008]. The cells were passed 48 hours prior to their passage. The stage I differentiation of these cells were set up in six 96-well round bottom, plates - 100 µl volume/well, 3000 ES cells/well, 60 wells of EBs per 96-well plate in Bovine Serum Albumin (BSA) Polyvinylalchohol Essential Lipids **(**BPEL) media with cytokines.

Culture of BC1 IPSC: BC1 IPSC cells were kindly provided by Linzao Cheng. The cells were cultured in ESC media as mentioned previously. They were differentiated by stromal cell co culture on M210 and BC1 spin EBs were also set up as described above.

Stromal cell co-culture for differentiation: The mouse bone marrow stromal cell line M2-10B4 was grown in R-15 media – RPMI (Invitrogen) containing 15% fetal bovine serum (FBS) (Hyclone), 1% P/S, 1% NEAA, and 0.1 mM β-ME. M2-10B4 cells were inactivated with 10 ug/mL mitomycin C in M2-10B4-conditioned media for 3 hours at 37 °C with 5% CO2 prior to culture on gelatin-coated plates.

Culture of CD73 $^{+}$ MSCs: Sorted $CD73^{+}$ cells were plated onto gelatin-coated plates and grown in MSC media containing alpha-MEM (Invitrogen) supplemented with 10% FBS, 1% P/S, 1% MEM-nonessential amino acids, 2 mM L-Glutamine and 0.1 mM β-ME. Media changes occurred every 2–3 days. Adherent cells were selected for during culture expansion. The cells were passaged by enzymatic dissociation upon reaching 80–90% confluence.

Mesenchymal differentiation by stromal co-culture: Mesenchymal differentiation of hESCs H9 p77 occurred after culture on stromal cell layers. hESCs were passaged onto M2-10B4 mouse stromal cells with differentiation media consisting of RPMI supplemented with 15% defined FBS, 2 mM L-glutamine, 0.1 mM β-ME, 1% NEAA, and 1% P/S. Media was changed every 2–3 days. Time point analyses were made at Day 10, 12, 14, and 17. hESCs were made into a single cell suspension by treatment with Collagenase IV (1 mg/mL) (Invitrogen), followed by 0.05% trypsin/EDTA (GIBCO/Invitrogen) supplemented with 2% chick serum (Sigma) and utilized for FACS analyses.

Magnetic sorting for CD73 population and Flow cytometry analysis:-

On day 21 the H9s on M210 were sorted for a CD73 positive population by Magnetic sorting PE selection kit. The cells were harvested after a Collagenase IV / 0.05% Trypsin treatment. The cells were Collagenase IV treated for 5 minutes, centrifuged and resuspended in Trypsin with 2% chick serum. This was incubated at 37deg C for 5 minutes and vortexed every minute to dissociate the cell pellet. The trypsin was quenched with equal amount of FBS containing media (R15 media) and centrifuged at 1500 rpm for 5 minutes. The cell pellet was resuspended in fresh R15 media and passed through a 20um filter to make a single suspension of cells. The H9s in spin EB stage 1 was dissociated on Day 8 using Trypsin EDTA/Chick serum enzymatic dissociation. The cells were made into a single cell suspension by filtration. A part of the "pre-sort" single cell suspension was aliquoted for Flow cytometry (FACS) analysis. The cells were stained with Isotype IgG1 PE antibody and CD73 PE antibody (BD Biosciences). The rest of the cells were centrifuged and resuspended in 200 μL of bead buffer (2% FBS,0.05mM EDTA in PBS) for enrichment of a CD73 positive population. Antibodies for PE

conjugated with magnetic beads were used to select CD73+ cells from single cell suspension using the EasySep PE selection kit (Stemcell Technologies). Post-sorted CD73+ cells were plated under MSC conditions on a 75 cm^2 flask (BD Falcon). A portion of the post-sort was aliquoted for FACS analysis to look at typical MSC phenotypic markers. The different sample sets were CD34 APC/CD73 PE, CD90 PE/CD 105 APC, CD44 APC/ CD146 PE, and Flk-1 PE (all the antibodies from BD Biosciences). The isotype control used was IgG1 APC/PE double staining. The phenotype of the post sort MSCs was monitored for 4-5 passages, after which they were directed into the trilineage differentiation.

MSC trilineage differentiation: The hESCs differentiation on M210 and the D8 spin EB dissociated Stage I cells, sorted for a CD73 population underwent mesenchymal differentiation for osteogenic, chondrogenic and adipogenic differentiation. Cells were plated on culture dishes prepared with 0.1% gelatin and cultured in osteogenic supplemented media for up to 4 weeks. Osteogenic supplemented media was α-MEM supplemented with 10% FBS, 2 mM L-glutamine, 1% NEAA, 1% P/S, dexamethasone (0.1 μM), β –glycerol phosphate (10 mM), and ascorbic acid (50 μg/mL). Cells were plated on culture dishes prepared with 0.1% gelatin and cultured in adipogenic supplemented media for up to 4 weeks. Adipogenic supplemented media was α- MEM supplemented with 10% FBS, 2 mM L-glutamine, 1% NEAA, 1% P/S, dexamethasone (0.5 μM), isobutyl xanthine (0.5 μM) and indomethacine (60 μM). Cells (2.5×105) were spun into a pellet and cultured in a conical tube in chondrogenic supplemented media for up to 4 weeks. Chondrogenic supplement media was α-MEM supplemented with 10 % FBS, L-glutamine, NEAA and P/S, TGF-β (10 ng/mL) and ascorbic acid (45 μg/mL). After 4 weeks, osteogenic, adipogenic and chondrogenic cultures were fixed with 10% formalin and stained with von Kossa, Oil Red O or Alcian Blue staining, respectively. Chondrogenic cell pellets were embedded in paraffin wax, and sectioned. Stained cultures were imaged on a microscope.

Culture of IPSC: IPSC were cultured in the same method as indicated above for the hESCs. Initial passages were subject to ROCK (Rho-associated protein kinase) Inhibitor treatment (10 μM, EMD Biosciences) during passage of cells onto a fresh layer of MEFs.

Culture of Neonatal Human Dermal Fibroblasts – NHDFs passage 6 were thawed and plated out at 1 M / T75 flask. The cells were fed with fibroblast media (DMEM high glucose with standard FBS) every other day. The cells were harvested when they reached 90% - 95% confluence.

Culture of PBMCs for STEMCCA Flox Lentivirus transduction:

Peripheral blood was collected using standard venipuncture. Blood was processed by Ficoll (GE Healthcare) gradient method and the buffy coat of mononuclear cells was isolated. The PBMCs were cultured in RPMI, 10% FBS and 2.5 mg/mL pHA (200 ug/mL) or DMEM-High Glucose supplemented with 10% FBS, 100ng/mL SCF, 20ng/mL Flt3L, 100ng/mL thrombopoietin (TPO), 20ng/mL IL-3, 100ng/mL IL-6, 20ng/mL granulocyte macrophage colony stimulating factor (GM-CSF), and 50ng/mL macrophage colony stimulating factor (M-CSF) on fibronectin coated NUNC plates.

Human PBMC culture for Reprogramming by Episomal Vector Transfection

The PBMCs isolated from patient blood samples were cultured in Serum free media (SFM) (50% IMDM and 50% Ham's F-12) supplemented with SCF (50 ng/ml), IL-3 (10 ng/ml), EPO (2 U/ml, R&D Systems), IGF-1 (40 ng/ml), Synthecol (500X, Sigma), Linoleic and linolenic acid (1 mg/mL, Sigma), ITS (Invitrogen), Ascorbic acid (5 ng/mL), 2 mM L-Glutamine and dexamethasone (1 uM, Sigma). Another condition used for culture alpha-MEM media containing 10% FBS and 10 ng/mL IL-7 (Peprotech). The media was replenished on day 3 and 6.

Production of STEMCCA Flox LV:

2nd-generation Lentivirus was produced using polyethylenimine (PEI) co-transfection. Prior to transfection, 150cm^2 flasks (BD Falcon) were coated with poly-L-lysine (0.001%, Sigma). Poly-L-lysine was removed and the flasks were allowed to dry. 293s (Human Embryonic Kidney 293 cells**)** were grown to confluence in 150cm^2 tissue culture flasks. For one 80% confluent flask of 293s, 10.5 ug total plasmid DNA composed of vector ΔNRF, and pMD2.G was added together in a 3:2:1 ratio with 150mM NaCl to 1mL final volume and incubated at room temperature 5 minutes. 84 uL PEI solution (7.5mM) (Sigma) was added to 150mM NaCl to 1mL and incubated at room temperature

5 minutes. 1 mL NaCl/plasmid solution was added to 1mL NaCl/PEI solution and incubated at room temperature for 10 minutes. Meanwhile, the confluent flasks were trypsinized and the culture resuspended into 20 mL MEF media per flask. For a single flask, after the 10-minute incubation, the 2mL solution was added to the 20mL 293 culture. This culture was plated into the previously poly-L-lysine-coated flasks. Supernatant was collected at 24 hours and 36 hours. Supernatants were filtered through a 45μm filter (Nalgene) and then carefully balanced in a laminar flow hood before being centrifuged for 1.5 hours at 23,900 rpm in an ultracentrifuge. The viral pellets were resuspended at 4°C overnight on an oscillator in 300 uL PBS. Aliquots were frozen down at -80°C. Virus titer was determined using the Enzyme RT Assay kit (Invitrogen) and it was found to be $1.65*10^8$ pfu/mL

Infection of NHDF with SC LV – We infected 2 wells of NHDF p7 fibroblasts of 125000 cells each with StemCCA flox LV. The volume of the virus required for the infection was calculated based on the virus titer and number of cells for infection and also the desired MOI of infection.

Volume of virus required = <(No of NHDF cells/mL) * MOI>/ Virus titer

= <(125,000/2.5 mL) * 25> / (1.65*10^8 pfu/mL)

= 7 uL virus.

A master mix solution of the fibroblast media and the virus was made – for 2 mL fibroblast media, 2 uL of 5 ng/mL polybrene and 14 uL of the virus (Virus aliquot was thawed on ice) was added. 1 mL of the master mix was added per well. The wells were incubated at 37 °C.

Infection of PBMC with STEMCCA LV Flox:

PBMCs were transduced with STEMCCA LV Flox of MOI = 25. The virus titer was $1.65*10^8$ pfu/mL. The volume of virus required to transduce 500,000 cells / well was calculated as explained previously. A master mix solution with the virus, polybrene in PBMC media was formulated and added to fresh fibronectin coated wells.

Nucleofection of PBMCs with the episomal vectors:

2 million PBMCs were nucleofected per reaction with 5 µg of pEB C5 and 5 µg pEB TAg plasmid DNA. Amaxa's CD34 Nucleofector kit – Program T-016 was used [Chou et al., 2011]. The nucleofected PBMCs were plated onto fibronectin-coated plates. PBMC media was changed every day, for three days.

Post transduction Culture of NHDF in fibroblast condition and transfer to feeders:

After viral transduction of NHDF cells with STEMCCA Flox LV, the media was changed after 12 hours and every day for 5 days. The cells were maintained in fibroblast condition for this period. The cells were transferred to double density MEF feeder plates on Day 6. The virus transduced fibroblasts were harvested with 0.05% Trypsin treatment and counted. 50000 cells were transferred per well of 2X density MEF 6 well NUNC plate. We had 4 such plates. The media was changed to hESC cell media with 10 µM ROCK Inhibitor. 3 mL of media was given to each well, and media was changed every other day.

Post nucleofection culture of PBMC and transfer to feeder layers:

After 3 days of culture in PBMC media – both SFM 17 and alpha-MEM/IL-7 conditions, the PBMCs were counted and transferred to high density feeders at 100,000 cells per well and cultured in IPSC media. The cells were started on conditioned IPSC media after a week of culturing on feeders.

Picking IPSC colonies – IPSC-like colonies were observed from Day 12. These colonies exhibited a typical cobblestone appearance. ROCK Inhibitor treatment was continued until Day 26, when these colonies were picked and transferred to single density feeders, a colony per well of a 6 well plate. The cells were fed with regular ES cell media; ROCK Inhibitor treatment was used when the cells were passaged onto feeders.

Immunostaining of NHDF IPSCs: Undifferentiated ESC and iPSCs were washed once in PBS, fixed in 4% paraformaldehyde (PFA) for 15 minutes, the cells washed twice with PBS, washed twice with PBS-Tween20 (PBS-T, 0.1% Tween20 in PBS). The cells were permeabilized with 0.1% Triton-X-100 in PBS (Fisher Scientific) for 45 minutes, washed twice with PBS-T, blocked for 90 minutes in blocking buffer (5% BSA in PBS), washed once with PBS-T, and covered in a solution containing various amounts primary

antibodies diluted into blocking buffer (OCT4-specific mouse anti-human monoclonal antibody, diluted 1:250, from Millipore; TRA-1-81-mouse anti-human monoclonal antibody, diluted 1:500, from Millipore; SOX2- mouse anti-human monoclonal antibody, diluted 1:500; NANOG-specific goat anti-human monoclonal antibody, diluted 1:500; SSEA-4-mouse anti-human monoclonal antibody, diluted 1:500 (from R&D Systems unless mentioned otherwise). Cells were incubated with the primary antibody overnight at 4°C. After 18 to 24 hours, the cells were washed twice with PBS-T, and Alexa Fluor 488-conjugated goat anti-mouse or donkey anti-goat secondary antibodies (Invitrogen) diluted 1:500 in blocking buffer were added to respective wells and the cells were incubated in dark for one hour. The cells were counterstained with DAPI (1:1000) for about ten minutes at room temperature in dark. The cells were washed twice in PBS and imaged using a fluorescent microscope (Zeiss).

Results:

Characterization of Mesenchymal Stem Cells:-

hESCs have been established to make MSC that were characterized by the expression of surface markers CD73, CD 90, CD105, CD 44 and CD 146 and the lack of specific hematopoietic markers. However the more stringent test for characterization of MSCs is to measure their ability of trilineage differentiation into bone, cartilage and adipose tissue.

Our main idea was to compare the conventional stromal co-culture method of differentiation with the "spin EB" method used for hematopoietic differentiation. The spin EB method of differentiation was originally published by Elefanty's group and it was clear that this system had advantages in terms of precise cell numbers needed for an embryoid body, reproducible uniform size of each EB and used of defined media [Ng ES et al., 2008]. We have used the spin EB method to generate hematopoietic cells on a regular basis. It was then decided to extend this method to derivation of MSCs.

Derivation of MSCs:

The hESC or IPSCs were co-cultured on M2-10B4 (mitogen inactivated M2-10B4-b mouse stromal line) for 21 days in R-15 media and the cells are harvested and magnetically sorted for a CD73 expressing population which are now termed as "MSC"s. The MSCs are cultured in Alpha MEM/ 10% FBS conditions on gelatin coated plates and are seen to have a typical fibroblast-like morphology. After 4-5 passages, the cells are directed towards trilineage differentiation in the appropriate media conditions.

We set up a stromal co-culture differentiation of hESC line H9 (passage 72). On day 21, the cells were sorted for a CD73 positive population by magnetic sorting. The sample cell population had about 50% of CD73 positive cells. The cells were plated on a 25 cm^2 gelatin coated BD Falcon flask in MSC media conditions. The cells were passaged enzymatically upon reaching confluence and characterized with Flow cytometry. These cells were CD73 positive, CD90 and CD105 double positive population (>90%). On

passage 5, the cells were harvested and after analysis with FACS (Fig 1a), were directed into osteogenic, adipogenic and chondrogenic differentiation.

Trilineage differentiation of MSCs:

At the end of 28 day differentiation, the osteogenic and the adipogenic cells were fixed with 4% PFA for staining. Von Kossa staining was performed on the cells in osteogenic conditions to confirm the presence of osteocytes by measuring their calcium deposits. The adipocytes were detected by Oil Red-O staining that stained lipid globules in the cultures. Following a 31 day differentiation period, the chondrocytes pellets were fixed in formalin initially followed by 70% Ethanol and sent to the Masonic Cancer Center Comparative Pathology Shared Resource facility, University of Minnesota (St Paul) to be paraffin embedded, sectioned and stained for Alcian Blue. The Alcian blue stain confirms successful chondrogenic differentiation by detecting the glycosaminoglycans and mucopolysachharides in the extra-cellular matrix of chondrocytes. Our H9s cells on M2-10B4 successfully gave rise to a CD73 positive MSC population, with the appropriate expression of characteristic surface markers as analyzed by flow cytometry, and also gave rise to bone, cartilage and fat tissue precursors in culture. (Fig 1b)

Derivation of putative "MSCs" from H9 spins EBs:

H9 cells were TrypLE (enzymatic dissociation) adapted for 24 passages. A well of these H9s was harvested, and 6 plates of spin EBs were set up. On Day 8, the spin EBs were dissociated with 0.05% Trypsin/EDTA with chick serum treatment, and the cells were filtered (70μm) into a single cell suspension. A portion of this was aliquoted for flow cytometry analysis to look at the percentage of CD73 expressing population. The cells were sorted for a CD73 positive MSC population as explained previously. The spin EB derived cells had a good enrichment of the CD73 population, comparable to the H9s on M2-10B4. The cells were cultured in MSC conditions similar to the H9s on M2-10b as described earlier. They were phenotypically analyzed by flow cytometry for the expression of typical MSC markers (Fig 1a). Post 5 passages, they were directed towards

osteogenic, adipogenic and chondrogenic differentiation. At the end point of differentiation, the cultures were analyzed as previously described (Fig 1b)

H9 spin EBs derived putative MSCs do not differentiate into bone, cartilage or adipose tissue:

The results of the differentiation of MSCs derived from H9s using a spin EB protocol was interesting in that although the putative MSCs expressed all the characteristic markers (Fig 1a), they did not make osteogenic and adipogenic precursors in culture (Fig 1b). The Von Kossa and the Oil-Red O staining on the MSCs in the respective conditions returned negative results. The MSCs in chondrogenic conditions did stain positive for Alcian Blue but at a much lower level compared to their H9/M2-10B4 counterparts, raising questions about the validity of the chondrocytes. This seemed to indicate to us clearly that the spin EB method of differentiation although pushes the cells towards a mesodermal lineage, does not clearly favor a mesenchymal lineage.

Role of cytokines in Mesenchyme induction:

We postulated that the above findings could be a result of the nature of cytokines used in the BPEL media for differentiation in the spin EBs. The three cytokines used in the Stage 1 BPEL media are Stem cell factor (SCF), VEGF (Vascular Endothelial Growth Factor) and BMP4 (Bone morphogenetic protein 4). Stem Cell factor is a cytokine that plays an important role in hematopoiesis and has known to regulate hematopoietic stem cells (HSCs) in their niche in bone marrow. VEGF, as the name suggests, induces vasculogenesis and angiogenesis during embryonic development [Pick et al., 2007, Lohela et al., 2009]. BMP-4 on the other hand, is a TGF-beta superfamily ligand that plays an important role in formation of mesenchyme [Zhang et al, 2008]. So we decided to induce formation of spin EBs with BMP 4 alone in the BPEL media, and compare it to the control H9s that received all three cytokines. The spin EBs were dissociated as stated in our protocols, however we observed that the spin EBs in the BMP 4 only group were poorly formed, and lesser number of spin EBs were formed per 96 well plate. The cells also did not survive the enzymatic dissociation that is used to produce a single cell

suspension for magnetic sorting for a MSC population. The CD73 positive populations in both samples were comparable, and the BMP4 only spin EBs did not show an increase in mesenchymal population as we expected (Fig 2). Thus we concluded that SCF and VEGF although are not essential for a mesenchymal specification play a quintessential role in mesenchymal patterning as such and also the production of embryoid bodies. Our findings agreed with those of Elefanty's group [Pick et al., 2007] that BMP4 alone is insufficient to promote a mesodermal lineage in embryonic stem cells by the spin EB method.

BC1 iPSC derived MSCs – Stromal co-culture v/s spin EB method:

We next tested if our results of M2-10B4 differentiation supremacy over spin EBs were a collective phenomenon observed in all pluripotent cells or if it was a mere reflection of the initial population of cells used. The same experiment of M2-10B4 versus spin EB method of differentiation was set up with an induced pluripotent stem cell population – BC1 IPSC (Fig 3a). The preliminary results obtained were a confirmation of our previous findings - while, BC1 IPSCs on M2-10B4s made a good MSC population that made adipocytes (Fig 3b i) and chondrocytes (Fig 3b ii), the CD73 positive putative MSCs from spin EBs although express characteristic MSC markers, with increasing passages, did not exhibit typical MSC morphology (Fig 3b iii) or expand sufficiently to give us a sufficient initial population for the trilineage differentiation.

Generation of iPSC from PBMCs:-

Our lab has routinely made iPSCs from a variety of sources. We have established iPSC lines from NHDF, dental pulp cells, and umbilical cord blood. Our goal was to then produce IPSC from blood. As described in detail, blood cells are easier to obtain than fibroblasts, and we can isolate millions of PBMCs from a relatively small volume of blood drawn. Thus, production of patient specific iPSCs seemed an achievable milestone.

Attempts to generate IPSC from PBMC using STEMCCA LV:

We decided to use the STEMCCA lentivirus cassette, a single LV vector encoding the four transcription factors - Oct4, Klf4, Sox2, and cMyc (Fig 4a). Combining the four factors into a single transcript was stated to be an efficient reprogramming method and permitted derivation of iPSCs with simply a single viral integration [Sommer et al., 2009]. Dr. Minh Hong in our lab had already established a NHDF- IPSC line using the same vector; however, attempts to reprogram PBMCs to IPSC using the same did not yield results. Another paper from the same group reported that using an excisable polycistronic lentiviral vector leads to efficient derivation of iPS cells free of exogenous reprogramming transgenes [Sommer et al., 2010]. The cassette encoding the 4 reprogramming factors was flanked by loxP sites, which would enable Cre- mediated excision of reprogramming transgenes following the generation of IPSC colonies. Thus we decided to reprogram PBMCs with the STEMCCA Flox vector.

Initial attempts of reprogramming were carried out with NHDF cells as a proof of principle that vector was proficient in making IPSCs (Fig 4b). NHDF p6 cells were transduced with STEMCCA Flox LV of MOI = 25. The cells were maintained in fibroblast media for 6 days post transduction, and then transferred to high density MEFs and cultured in ES media. The media is changed every day and colonies started appearing by Day 14. We picked the colonies exhibiting typical ESC – cobblestone morphology and passed them onto normal density feeders with ROCK Inhibitor treatment. By Day 26 we passed the IPSC colonies from our initial passage onto normal density feeders. The cells were characterized by Immunostaining for pluripotency markers (Fig 4c). Further validation is required in terms of differentiation assays and teratoma formation to characterize our IPSC line but we agreed that our experiments were sufficient for our purpose.

We proceeded to attempt transducing PBMCs with our LV construct. PBMCs were cultured in PBMC media conditions with cytokines for 6 days, before virus transduction. Post transduction with STEMCCA Flox LV, they are transferred to new fibronectin coated plates and cultured in PBMC media for 3 days. These are then harvested and plated onto high density feeders and cultured in ES media (Fig 5). After a week of

culture, MEF- conditioned media is used for culturing the cells. We noticed that post 3 weeks of culture the PBMCs fail to produce any ES-like colonies. Fearing our technique, we repeated the experiments with multiple duplicates, however not yielding any results.

Attempts to generate IPSC from PBMC using episomal vectors:

Cheng used integration-free episomal vectors and reprogrammed peripheral blood to IPSCs [Chou et al, 2011]. This method was ideal as it was virus and integration –free and also, by the reports in the paper much more efficient than small molecules or miRNA used for reprogramming. Two plasmids were used in the experiment - the pEB C5 (Fig 6a) which contained the four reprogramming factors and the pEB TAg, which encoded the SV 40 T-antigen that has been found to increase the efficiency of reprogramming 50-fold.

We obtained the plasmids from Add-gene and extracted plasmid DNA from the bacterial cultures. Test digestion with specific restriction enzymes and the results obtained confirmed the authenticity of the plasmids. The PBMCs were cultured in two media conditions – a serum-free media with cytokines and alpha-MEM with FBS and IL-7. The cells were prepped for 6 -8 days and 2 M cells were nucleofected with a total of 10μg of plasmid DNA as per the protocol from Cheng's group (Fig6b) [Chou et al, 2011].

There, however was considerable cell death post nucleofection. Only about 0.05 % of cells survive the nucleofection reaction (as determined by exclusion of Trypan Blue dye). The cells were transferred onto fibronectin coated plates post nucleofection and maintained in respective PBMC media condition for 3 days. The cells are then transferred onto high density feeders at 100,000 cells / well and maintained in ES media (Fig 3a).

The use of episomal vectors did not yield IPSC colonies by Day 14 post nucleofection as previous publications suggested [Chou et al., 2011]. We hypothesized that the reason could be possibly due to the large size of each of the two plasmids used – pEB C5 is 17 kbp and pEB Tag is around 12 kbp. This was probably resulting in almost negligible uptake of the plasmids by the PBMCs. The cell culture conditions could also possibly have influenced our results. The original publication by Cheng did in fact report that the

peripheral blood cells reprogrammed at 0.0007 % efficiency which is 50- fold lower than that for cord blood cells.

Our current test of nucleofection of NHDF cells with EBV plasmids show early positive results. Therefore we have confirmed that the episomal vectors are capable of reprogramming somatic cells. We are currently working on optimizing our experimental variables to generate IPSC from peripheral blood cells using these episomal vectors.

Discussion:

There were two components to this project. It aimed at optimization of differentiation protocols of inducing hESCs and IPSCs into functional $CD73^+$ MSCs that are capable of differentiation into bone, cartilage and adipose tissue. Secondly, it aimed at generation of iPSCs from somatic cells which could potentially be utilized to generate functional MSCs. The MSCs could serve as an unlimited reservoir of cells that could be used in either autologous or allogeneic repair of damaged bone, or cartilage tissue.

MSCs were derived from pluripotent stem cells and characterized by their ability to make bone, cartilage and adipose tissue. Attempts to define the role of differentiation techniques to derive MSCs from pluripotent stem cells are well underway. We clearly established that the conventional stromal co-culture exhibits supremacy over the new spin EB method of differentiation for production of a mesenchymal lineage. However more experiments are required to determine the role of cytokines in MSC development in the spin EB method. Use of TGF-beta and Wnt signaling proteins in Stage 1 of spin EB set up may tilt the kinetics towards MSCs than a hematopoietic lineage. We aim to establish such conditions with a H9 control receiving the standard three cytokines in near future. MSCs generated in this manner could be characterized further with gene array analysis looking at expression of "mesenchymal" markers at various stages of differentiation in all the sample sets to determine which condition best supports the generation of the CD73 expressing MSC population that is capable of trilineage differentiation. The cells in osteogenic and adipogenic conditions could also be analyzed in a similar manner to understand the mechanics of behavior of the samples at an epigenetic level.

iPSCs were successfully generated from fibroblasts using the STEMCCA Flox LV cassette. Our attempts to generate IPSCs from PBMCs using the STEMCCA vector failed to produce results. However although our current attempts using the integration-free episomal vectors have not given rise to preliminary success, we soon hope to optimize culture conditions, amount of plasmid DNA nucleofected and use of Sodium butyrate, also possibly small micro RNAs to yield results. It would also be ideal if we could arrive at a system of selecting for peripheral blood cells that have are successful in uptake of

both the plasmids. We are also looking at other methods of generating IPSCs in the near future. It has been reported that Sendai virus (SeV), a non-integrating RNA virus vector, efficiently generated human iPSCs from human fibroblasts and human blood cells and this holds great promise [Ban et al., 2011].

Figure Captions:

1) A) Phenotype of H9 CD73 + MSCs derived by stromal differentiation versus H9 spin EB derived MSC – The MSCs were analyzed by flow cytometry. MSCs from both populations showed expression of the typical MSC markers – CD73, CD90, CD105, CD44, and CD146.

   B) Trilineage differentiation of M210 versus sEB derived MSCs -The H9 MSCs were further differentiated in osteogenic, adipogenic and chondrogenic culture conditions and analyzed by Von Kossa, Oil-red O and Alcian blue staining respectively.

2) Effect of cytokines in mesenchyme induction in spin EBs – spin EBs that were in BMP4 only media versus the regular Stage 1 BPEL media were analyzed for their CD73 expression by flow cytometry.

3) A) BC1 IPSC derived MSCs by stromal co-culture vs. spin EB method

   Flow cytometry analysis – MSCs express CD73, CD90, CD105, CD44, and CD 146.

   B) i) Osteocytes derived from BC1/M210b MSCs – Von Kossa staining.

   ii) Adipocytes derived from BC1/M210b MSCs stained by Oil-Red O.

   iii) Chondrocytes derived from BC1/M210b MSCs with Alcian Blue staining.

   iv) Morphology of BC1 IPSC spin EB derived putative MSCs

4) A) Schematic of the STEMCCA Flox Lentivirus vector

   B) Schematic of generation of NHDF IPSCs

   C) Immunostaining of NHDF IPSCs for pluripotent markers

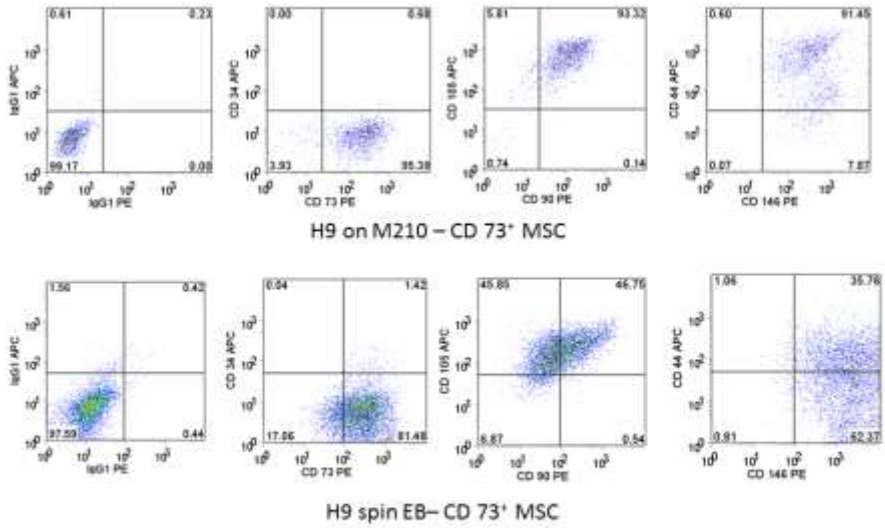
5) Generation of PBMC – IPSC using STEMCCA LV vector

6) A) Plasmid map of pEB – C5 (Chou et al., 2011)

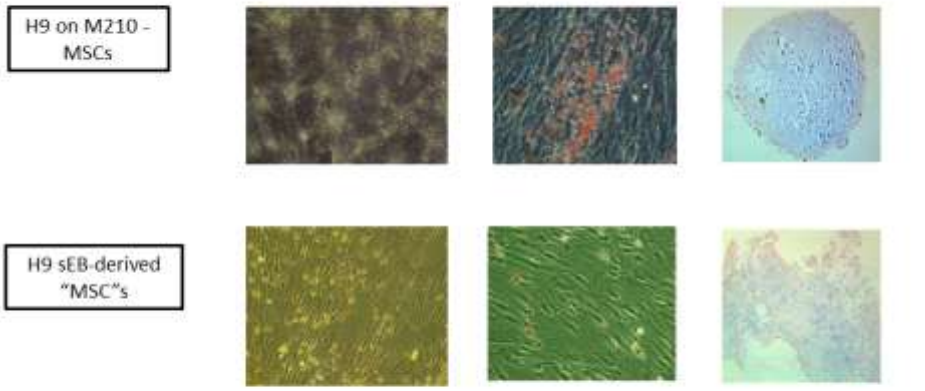
   B) Generation of PBMC –IPSC using integration-free episomal vectors

Figures:

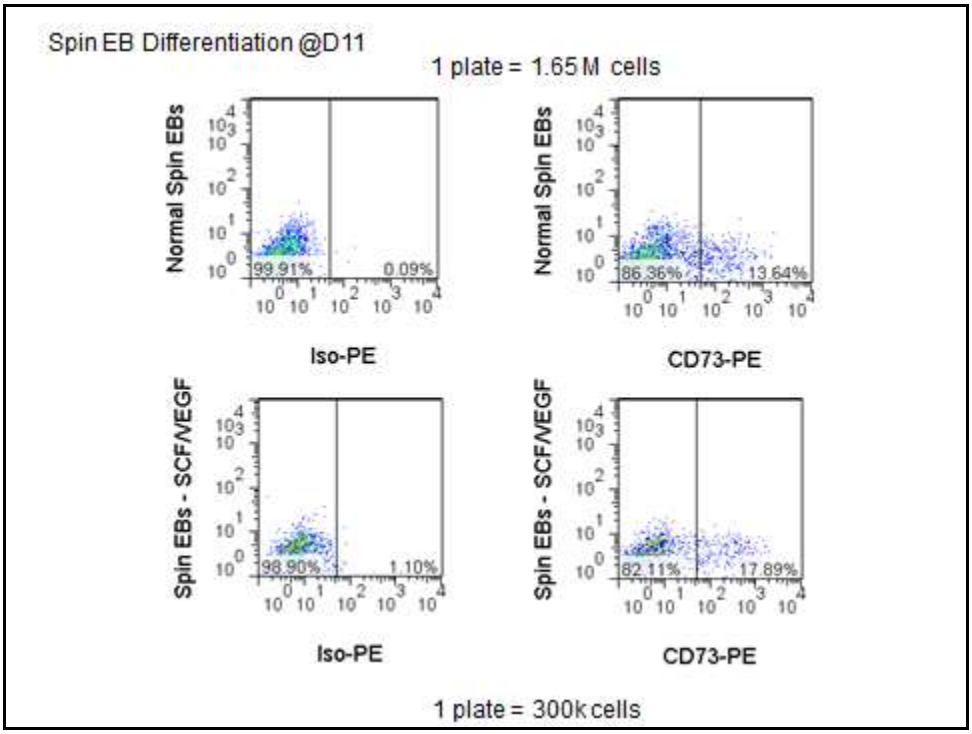
1a)



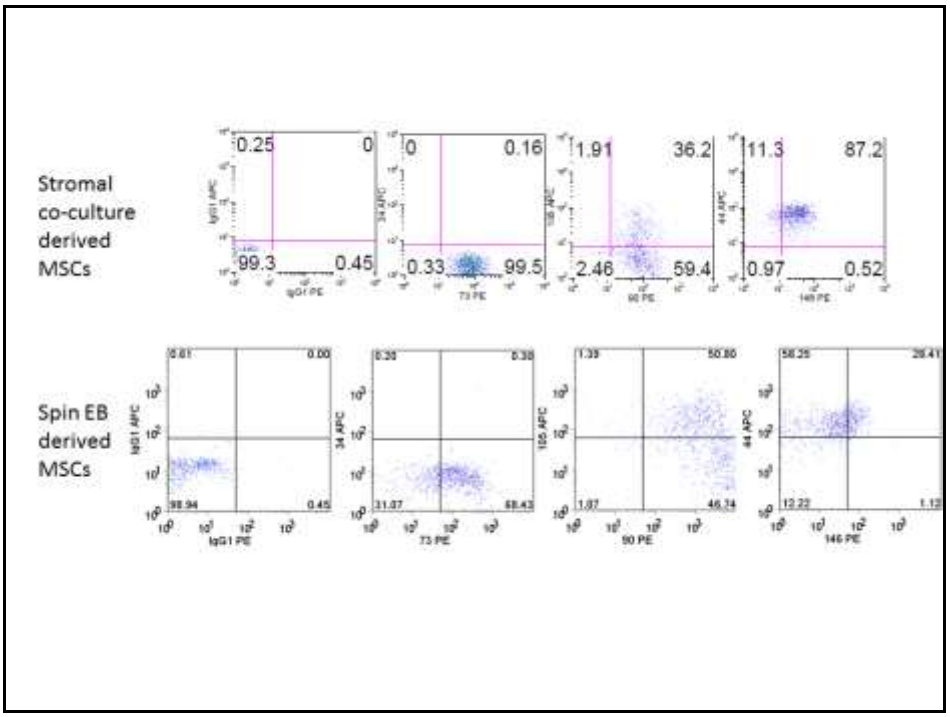
1b)



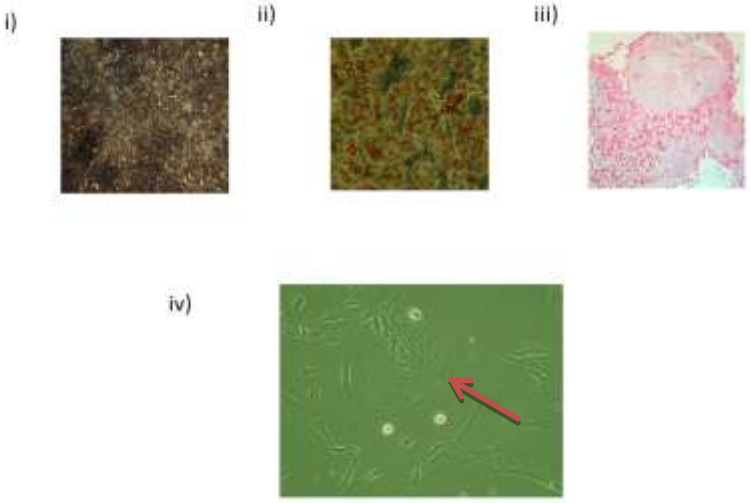
2.


Spin EB Differentiation @D11
1 plate = 1.65 M cells
Normal Spin EBs
99.91%
0.09%
Iso-PE
Normal Spin EBs
86.36%
13.64%
CD73-PE
Spin EBs - SCF/VEGF
98.90%
1.10%
Iso-PE
Spin EBs - SCF/VEGF
82.11%
17.89%
CD73-PE
1 plate = 300k cells

3a)


Stromal co-culture derived MSCs
0.25
0
99.3
0.45
0
0.16
0.33
99.5
1.91
36.2
2.46
59.4
11.3
87.2
0.97
0.52
Spin EB derived MSCs

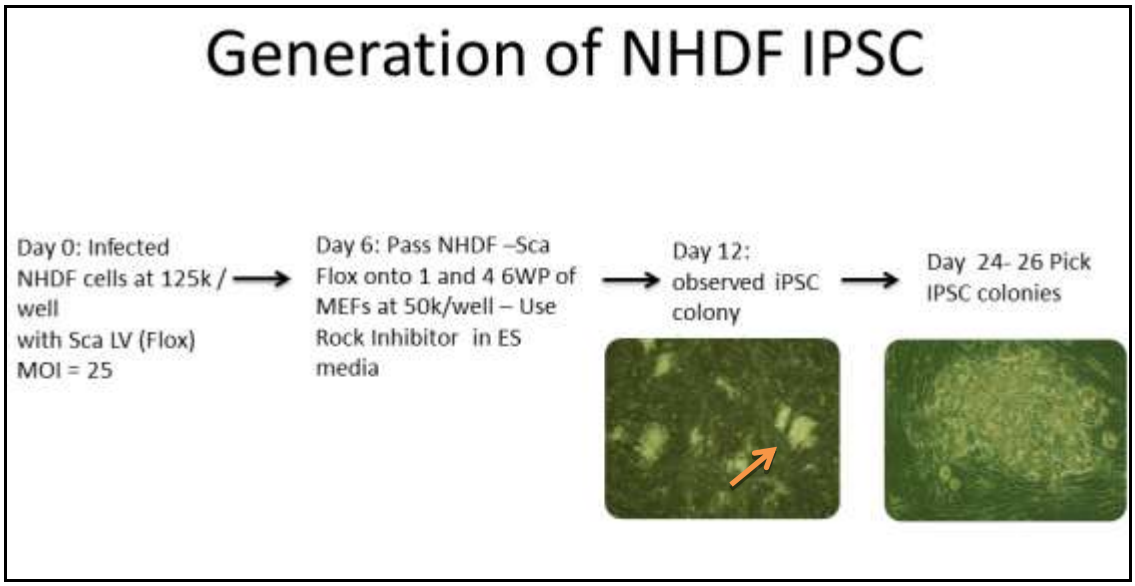
3b)


i)
ii)
iii)
iv)

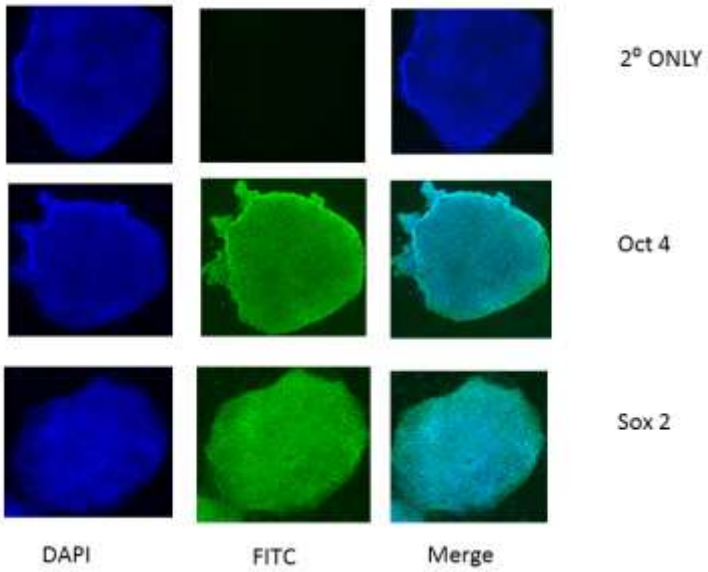
4a)

LoxP
5'-
UTR
EF1α
Oct4
2A
Klf4
IRES
Sox2
2A
cMyc
3'-
UTR

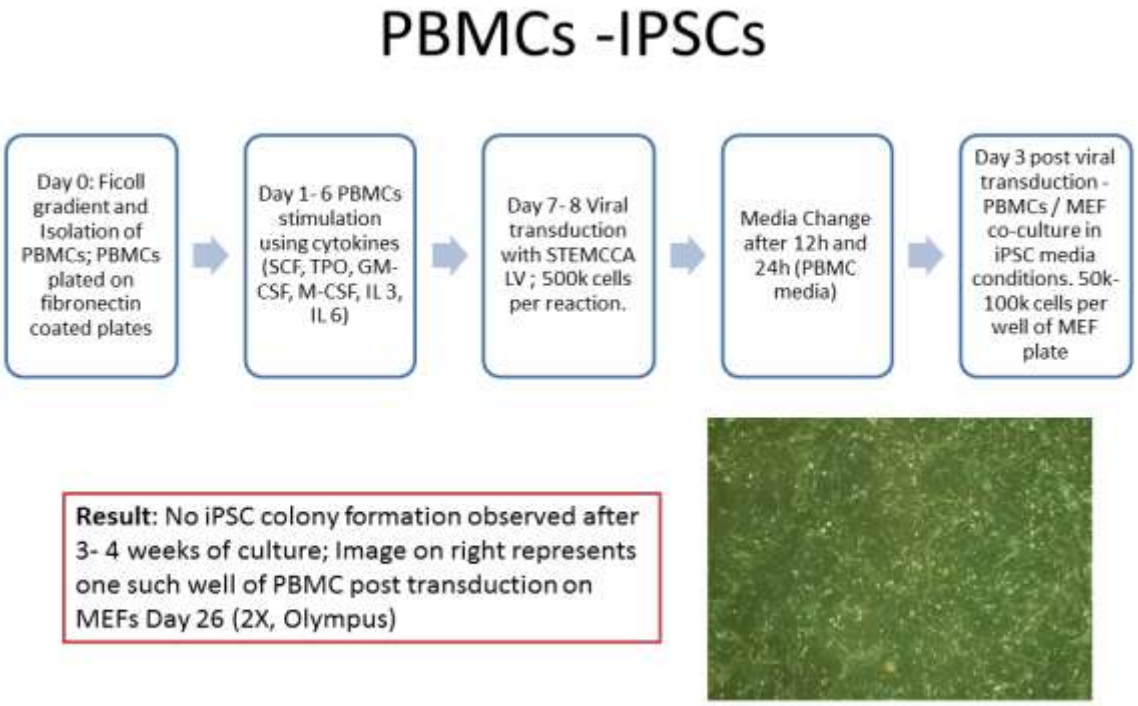
4b)


Generation of NHDF IPSC
Day 0: Infected NHDF cells at 125k / well with Sca LV (Flox) MOI = 25
Day 6: Pass NHDF –Sca Flox onto 1 and 4 6WP of MEFs at 50k/well – Use Rock Inhibitor in ES media
Day 12: observed iPSC colony
Day 24- 26 Pick IPSC colonies

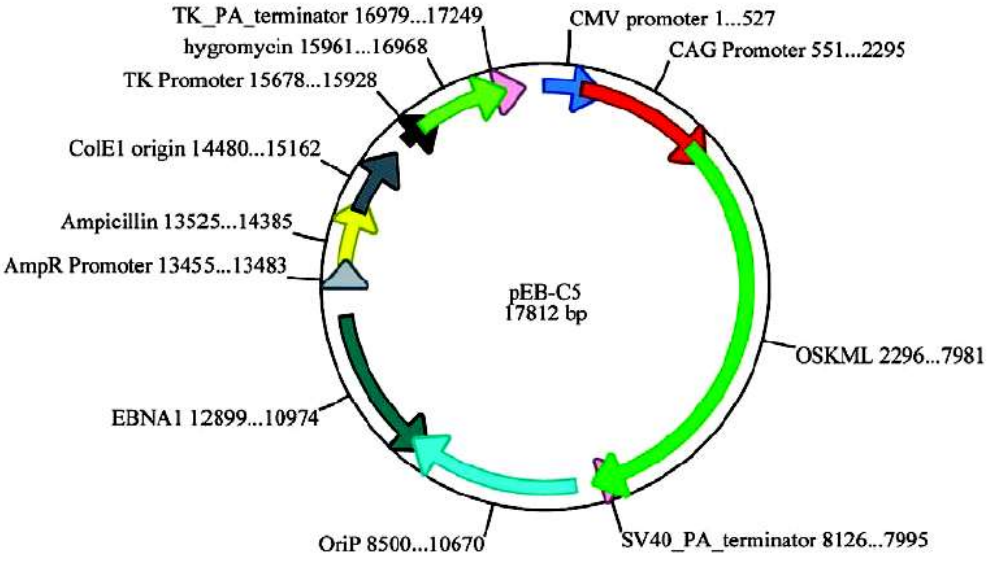
4c)


2° ONLY
Oct 4
Sox 2
DAPI
FITC
Merge

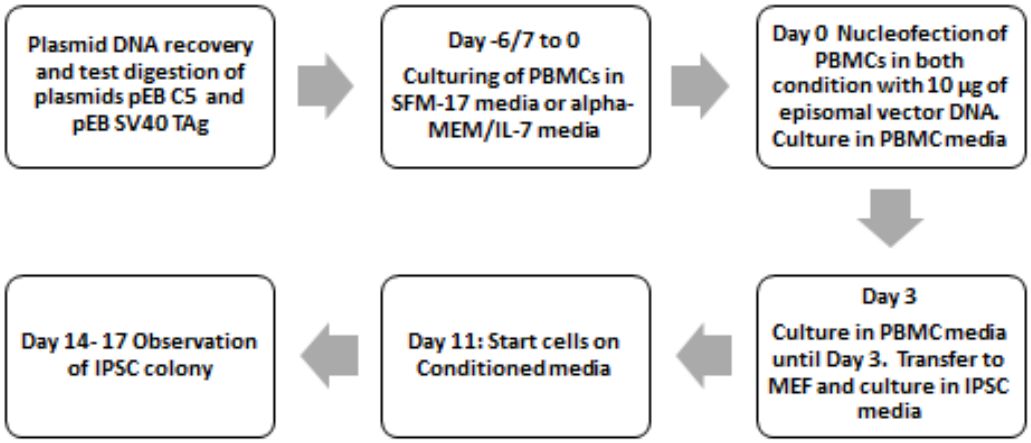
5)

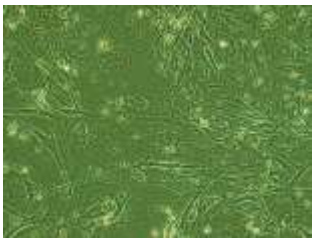

PBMCs -IPSCs
Day 0: Ficoll gradient and Isolation of PBMCs; PBMCs plated on fibronectin coated plates
Day 1- 6 PBMCs stimulation using cytokines (SCF, TPO, GM-CSF, M-CSF, IL 3, IL 6)
Day 7- 8 Viral transduction with STEMCCA LV ; 500k cells per reaction.
Media Change after 12h and 24h (PBMC media)
Day 3 post viral transduction - PBMCs / MEF co-culture in iPSC media conditions. 50k-100k cells per well of MEF plate
Result: No iPSC colony formation observed after 3- 4 weeks of culture; Image on right represents one such well of PBMC post transduction on MEFs Day 26 (2X, Olympus)

6a)



6b)





SFM-17 PBMCs in IPSC media – No colony formation

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