HEMATOPOIETIC DEVELOPMENT FROM ADULT HUMAN EMBRYONIC STEM CELLS AND INDUCED PLURIPOTENT STEM CELLS

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Abstract

The central goal of the project is to understand the role of non-coding RNAs in mediating cell fate decisions in both human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). Two specific aims are pursued in order to understand the process of hematopoiesis and erythropoiesis better. The first aim comprises of understanding the regulatory networks of hematopoiesis better, we plan to profile the microRNA and mRNA expression in hESC/iPSC-derived CD34+ CD45+ hematopoietic stem/progenitor cells compared to CD34$^+$ cells isolated from umbilical cord blood (UCB). The second aim deals with understanding the erythropoietic development, by profiling the long non coding RNAs (lncRNAs) from erythroid cells derived from hESCs and iPSCs. CD34$^+$ UCB derived erythroid cells are used as a positive control for comparing with hESC/iPSC derived erythroid cells. The overall significance of studying the role of non-coding RNA lies in the fact that it will help modulate the hematopoietic pathways and eventually be used for therapeutic purposes.

Table of Contents

List of Figures

<u>**Introduction**</u>

1. <u>**Human embryonic stem cells and Induced pluripotent stem cells**</u>

Human embryonic stem cells (hESCs) are cells derived from pluripotent inner cell mass cells of preimplantation blastocysts. They are pluripotent, have potential for unlimited self renewal and can produce teratomas that have derivatives from all three germ layers(1). *In vitro* differentiation of hESCs recapitulates events that occur during normal human embryogenesis(2,3). hESCs offer promising resources for future therapies due to their ability to generate mature cell populations, such as hematopoietic cells.

Another interesting population for future therapies and studies are the induced pluripotent stem cells (iPSCs). iPSCs are pluripotent stem cells made by reprogramming somatic cells (like fibroblasts) by expression of a few defined transcription factors(4). This expression is done using viral and other vectors. Yamanaka and group utilized Oct4, Sox2, Klf4 and c-Myc to derive first mouse and then human iPSCs(5). With advancement in studies, iPSCs can be successfully produced with transient expression of just one or two genes(6, 7). Human iPSCs form teratomas with contributions to all three germ layers (endoderm, ectoderm, and mesoderm) and have been utilized to produce many differentiated cell lineages. iPS cells were similar to human embryonic stem (ES) cells in morphology, proliferation, surface antigens, gene expression, epigenetic status of pluripotent cell-specific genes, and telomerase activity(8). iPSCs display more genetic and epigenetic abnormalities than do ES cells or fibroblasts- the cells from which they originated(9). While each has its own advantages and disadvantages, hESCs and iPSCs represent two pluripotent cell sources with far-reaching clinical potential in treating neurologic disorders, repairing or replacing damaged tissues, and producing transfusable blood components.

1.1 <u>**Hematopoiesis from hESCs and iPSCs**</u>

Hematopoietic stem cells (HSCs) located in the bone marrow replenish all mature cells within adult blood system(10). HSCs initially differentiate into multipotent progenitors (MPPs), further giving rise to Common myeloid progenitors (CMPs) and Common lymphoid progenitors (CLPs). CMPs eventually give rise to erythrocytes (RBCs), megakaryocytes (Mks)/platelets, monocytes and granulocytes while CLPs give rise to Natural killer (NK) cells, T cells and B cells.

Enriched blood populations have been generated *in vitro* by differentiating hESCs

and/or iPSCs to particular hematopoietic lineages. So, hESCs and iPSCs can serve as a substitute for primary cells for distinct clinical purposes(2) (Figure 1).

In vitro Hematopoietic differentiation of hESCs and iPSCs can be done by: (a) An embryoid body (EB) approach in which mechanically/enzymatically digested clumps of undifferentiated cells help provide a nidus for EB formation(11), (b) Differentiation of cells in monolayer culture(12), (c) Differentiation by co-cultivation of hESCs with stromal layer having lineage inductive properties(13). The Spin-EB method by the Andrew Elefanty group addresses a number of shortcomings of previously published methodologies by providing uniform culture conditions(14).

hESCs and iPSCs have been used in the past to derive the various mature blood cell lineages. Chang et al. derived erythroid lineage cells from hESCs and iPSCs(15). *In vitro* megakaryopoiesis and thrombopoiesis was first reported in 1995 using CD34$^+$ HSCs as a starting cell source(16). The first study to report the *in vitro* production of MKs from hESCs was published in 2006(17). Takayama et al. has reported the successful generation of both MKs and functional platelets from hESCs and from iPSCs(18). Slukvin et al were the first group to produce functional dendritic cells (DCs) from hESCs by using a 3-step differentiation protocol adopted from the mouse ESC system(19).

Differentiation into lymphoid lineage cells has been more difficult than myeloid lineage cells. Our lab has been the only group being able to successfully derive NK cells from human hESCs and iPSCs(20). To date derivation of other lymphocytes i.e. T and B cells from hESCs and iPSCs has been almost entirely lacking(21). While they have been produced *in vitro*, the method still remains inefficient. This is particularly surprising as T, B and NK cells are thought to arise from CLP cell i.e. the same starting population. Thus, significant gaps remain in understanding of the hematopioesis pathway.

2. microRNAs

Previous studies have shown that microRNAs (miRNA) to be playing a key role in development and regulation of hematopoietic cell lineages(22). MicroRNAs are a class of ~22 nucleotide long, non-coding small RNAs that influence mRNA stability and translation. miRNA genes are transcribed by RNA polymerase II as capped and polyadenylated primary miRNA transcripts (pri-miRNA). The RNaseIII enzyme Drosha

initiates the nuclear processing of the pri-miRNA into precursor miRNA (pre-miRNA) (23). The double-stranded RNA binding protein DGCR8 interacts with Drosha to form the microprocessor complex(24). Pre-miRNAs are exported from the nucleus by binding to the nucleocytoplasmic transport factor Exportin-5(25). Maturation of the pre-miRNA into an imperfect RNA duplex is mediated by the cytoplasmic enzyme Dicer. The strand of the duplex with the weakest base pairing at its 5' terminus is preferably loaded into the RNA-induced silencing complex (RISC)(26). The miRNA guides the RISC complex to the 3'UTR of target mRNAs. Association of miRNAs with their target mRNAs inhibits translation, but the exact mechanism of translational repression remain unclear due to the fact that both the initiation and elongation steps of translation are thought to be affected(27). There are hundreds of miRNAs found in a given mammalian genome, and a single miRNA can regulate hundreds of genes.

2.1 **Role of microRNAs in hematopioesis**

miRNAs thus act as master regulators of transcriptional programs, and their expression patterns are thought to reflect biological relationships between hematopoietic lineages(28). A growing body of work studying miRNA expression in abundant cell populations, including both normal and malignant cells, indicate that miRNAs play a critical role in hematopoiesis and blood cancer(29, 30, 31). Petriv et al. analyzed miRNA expression in single hematopoietic cells, which demonstrated that miRNA expression is very tightly regulated within highly purified populations(28).

Several miRNA species, including miR-130a, miR-196b, let-7d, and miR-125b, are generally upregulated in stem cell and progenitor populations, are not detected in any lymphoid cells, and are present in only a subset of myeloid cells. MicroRNAs are thus regulating hemtopoiesis by modulating multiple signalling pathways. Multifunctional role has been best characterized for miR-155 in myeloid and lymphoid cells(32). MicroRNA miR-21 regulates the metastatic behavior of B16 melanomal cells by promoting cell proliferation, survival, and migration/ invasion as shown by Yang et al.(33). Ooi et al. found that the anti-apoptotic effect of miR-125b is associated with development of lymphoproliferative disease, marked by expansion of CD8+ T lymphocytes(34). Early B cell differentiation is modulated by miRNA (miR)-150(35). miRNAs 17-5p-20a-106a direct human monocytes development(36) and miR-223 functions to restrict the granulocytic compartment *in vivo*(37). Since microRNAs play an important role in

hematopoiesis (Figure2), and they function by regulating mRNA pathways it is hypothesized that microRNA-mRNA pathways play an important role in the hematopoietic development. Studying these pathways will better define hESCs and iPSCs as a system that models normal human hematopoiesis. Additionally, these studies will allow better derivation of cell populations from hESCs and iPSCs that may be suitable for novel cell based therapies.

3. <u>Erythropoiesis</u>

The term erythropoiesis (erythro = RBC, and poiesis = to make) is used to describe the process of RBC formation or production. It is the pathway that produces mature blood cells from hematopoietic stem cells. During steady state hematopoiesis, approximately 10^{10} red blood cells are produced per hour in the bone marrow to maintain the hemoglobin level within fairly narrow limits. Production can be rapidly increased in the setting of ongoing blood loss or hemolysis.

The various stages of erythroid cell formation are as follows (Figure 3)(39):

Proerythroblast: Large cells, 15-20 micron in diameter, large nucleus with 2-3 nucleolei, nucleus basophilic with perinuclear halo and sometimes forms 'ear-shaped" bulges.

Basophilic Erythroblast: Slightly smaller cells, 12-17 micron in diameter, reduction of nuclear size disappearance of nucleolus, cytoplasm is moderately basophilic with high protein and RNA content, condensation of chromosomes occur.

Polychromatophilic Erythroblast: Diameter of cells is 12-15 micron, nuclear size is further reduced having a "cart-wheel" appearance, chromosomes are further condensed, cell division stops totally, cytoplasm is polychromatophillic, appearance of hemoglobin takes place.

Reticulocyte: Size of cells is 8 micron in diameter, formation of highly branched pattern by remains of mitochondria, ER, ribosomal RNA -hence named as reticulocyte.

Erythrocyte: Mature RBC which is 7.2 micron in diameter,biconcave shape,absence of nucleus,high hemoglobin content makes the cells typically pink red in color.

For massive *ex vivo* production of RBCs from hESCs and iPSCs the model system should satisfy three conditions:

(1)   The massive amplification of primitive HSC

(2)   The controlled induction of exclusive differentiation to the erythroid line.

(3)   The completion of terminal maturation to the stage of enucleated cells.

4

The model system can use embryoid body formation or stromal layer co-culturing. Cytokines

and stromal layer play an important role in formation of erythroid cells *in vitro*(40).

4. Long non-coding RNAs

Long non-coding RNAs (lncRNAs) are non coding RNAs greater than 200 nucleotides in length, transcribed by RNA polymerase II or III, and can account for nearly 60% of all non-ribosomal and non-mitochondrial RNA in human cells(41). These lncRNAs are involved in transcriptional silencing, chromatin remodeling and gene reactivation(42). LncRNAs constitute a significant fraction of the mammalian transcriptome. Compared to mRNAs, lncRNAs tend to be shorter and less well conserved at the primary sequence level. Expression of lncRNAs is often restricted to specific tissues and developmental stages, suggesting that many may regulate cell fate specification.

4.1 Role of lncRNAs in Erythropoiesis

LncRNAs are being characterized at a rapid pace. In hematopoiesis, the antisense lincRNA, HOTAIRM1, has recently been identified as an essential regulator of myeloid cell differentiation (43). Erythropoiesis is regulated at multiple levels by different factors to ensure the proper generation of red blood cells in response to various physiological and pathological stimuli. Wenqian Hu et al identified one erythroid-specific lncRNA, LincRNA-EPS, with potent anti-apoptotic activity. Expression of LincRNA-EPS is largely confined to terminally differentiating erythroid cells and its expression is induced in CFU-E (Colony forming unit-Erythroid) progenitors by Epo (Erythropoietin). Inhibition of this lncRNA blocks erythroid differentiation and promotes apoptosis(44). It is believed that looking at lncRNA expression in erythroid cells derived from hESCs and iPSCs will promote better understanding of the process of erythropoiesis as well as hematopoiesis. In the long run, it might will help improve the *in vitro* production of RBCs for transfusion.

Aim of Study

The study aims to understand the process of hematopoiesis and erythropoiesis better by :

    Specific Aim 1: Define profiles of microRNA and mRNA expression in hESC/iPSC-derived $CD34^+$ $CD45^+$ hematopoietic stem/progenitor cells to understand the miRNA

gene regulatory networks of hematopoietic development in these cells compared to CD34$^+$ cells isolated from umbilical cord blood (UCB).

Specific Aim 2: Define profiles of long non-coding RNA expression in hESC/iPSC-derived erythroid cells to understand long non-coding RNAs in erythropoiesis in these cells compared to erythroid cells derived from UCB.

Materials and Methods

Cell Culture:

Culture of human embryonic stem cells (hESCs): Human embryonic stem (H9 cell lines and H1 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 100,000 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 TrypLE treatment (GIBCO) and passed onto half density feeders i.e. 100,000 cells per well of a six well plate. The TrypLE 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 enzymatically (TrypLE, GIBCO)-adapted cells as described in the protocol by Elefanty's group in 2008(11). The cells were passed 48 hours prior to their differentiation. The Stage I differentiation of these cells was set up in six 96-well round bottom, plates - 100 µl volume/well, 3000 ES cells/well, 60 wells of EBs per 96-well round bottom low-attachment plate (NUNC, Thermo Scientific) in Bovine Serum Albumin (BSA) Polyvinylalchohol Essential Lipids (BPEL) media with cytokines. The Stage I EBs are transferred to the Stage II differentiation cultures in BEL media (Same as Stage I BPEL media but without polyvinylalchohol) in 24 well culture treated plates (NUNC, Thermo Scientific).

Culture of induced pluripotent stem cells (iPSCs): BC1 iPSCs were kindly provided by Linzao Cheng. Dub7 iPSC lines derived from cord blood selected for early hematopoietic progenitor marker CD34. The cells were cultured in ESC media as mentioned previously.

Setting up iPSC derived Spin EBs: Spin EBs were set up as described above for hESC lines.

Harvesting Stage I and Stage II EBs for FACS analysis: The StageI and Stage II EBs are dissociated using 0.05% Trypsin/EDTA (GIBCO) and chicken serum (Sigma) as described in the protocol by Elefanty's group(11). All cells are passed through 70-100µm cell strainer before running on FACS.

Flow Cytometry Analysis: Flow cytometry analysis is done for typical phenotypic markers at different time-points along the course of EB formation. The sample sets are CD34PE/ CD43APC, CD45PE/CD34APC, CD31PE/CD34APC, CD34PE/CD41aAPC (All antibodies from BD Biosciences). The isotype control used was IgG1PE/IgG1APC. The phenotype was monitored typically at Day7, 11, 13 and 18, though it varied at times with experiments. All analysis was done using FACS Calibur (BD Biosciences).

Magnetic Seperation of CD34$^+$ CD45$^+$ cells: Cells are harvested from EB plates as for FACS and then sorted for CD34$^+$ cells using the EasySep Human CD34 Selection Kit (using the purple EasySep magnet). The CD34$^+$ cells collected in the last step were then stained with CD45PE antibody (BD Biosciences). The cells were kept in the antibody for about 20 minutes, washed, and sorted using EasySep PE Selection Kit.

Processing of UCB Samples: UCB samples were transferred from collection bags to T150 culture flask (BD Falcon) using luer lock 60mL syringe. Initially 1mL heparin (Sigma) was added to the T150 flask before starting. Cord blood units were not mixed. Each donor is processed separately. Based on the oriiginal volume of UCB per bag, the sample was diluted 1:1 with IMDM medium (Thermo Scientific). Appropriate number of tubes with 20mL Ficoll-Paque Plus (GE Healthcare) were prepared. 30mL of diltued UCB on top of the Ficoll was slowly layered, and spinned for 30min, 1500rpm (room temprature, with NO brake). After spinning most of the medium from the top of each of the tubes was carefully aspirated taking care not to disturb the interphase, including any remaining medium left on top of the layer of Ficoll. The buffy coat layer was taken carefully and the bufffy coats from all the Ficoll tubes were pooled. The buffy coats were centrifuged at 1700rpm for 10 minutes at room temperature. After centrifugation,

the supernatant was aspirated off, the pellet was red. Washed with IMDM, combined all the pellets into one. Centrifuged at 1700rpm for 10 minutes at room temperature. After centrifugation, aspirated off the supernatant. Added ice-cold lysis solution i.e. Ammonium chloride to the pellets. Alternatively, ice-cold water was used for lysis. After adding the lysis solution, vortexed a little and kept on ice for 5 minutes. Added 10mL IMDM to the tube. At this point 100µL aliquot for FACS and 10µL aliquot for counting were taken. This is called the "pre-sort fraction". Centrifuged at 1700rpm for 10 minutes at room temperature. Aspirated off the supernatant, kept the pellet on ice. The pellets were white.

Magnetic Separation for CD34⁺ UCB cells: The UCB samples are then sorted for CD34⁺ hematopoietic progenitor cells using CD34 Microbead kit (Miltenyi Biotec). First, the CD34 cells are magnetically labeled with CD34 Microbeads. Then, the cell suspension is loaded onto a MACS column which is placed in the magnetic field of a MACS separator. The magnetically labeled CD34⁺ cells are retained within the column. The unlabeled cells run through, this fraction is thus depleted of CD34⁺ cells. After removing the column from the magnetic field, the magnetically retained CD34⁺ cells can be eluted as the positively selected cell fraction. The sorted samples are then collected and labeled as the "post-sort" fraction.

Flow Cytometry analysis of UCB cells: FACS analysis was done to determine the percentage of CD34⁺ cells in the post-sort fraction. The pre-sort fraction was stained with CD34 APC and CD45APC. The flow through fraction was also stained for CD34APC. The post sort fraction was stained with CD34APC. The Isotype control used was IgG1APC. The final percentage of CD34+ cells was determined and the post sort fraction was frozen using 10% DMSO, 30% FBS and rest IMDM.

miRNA Isolation: miRNA isolation is done using mirVana miRNA Isolation kit from Invitrogen. It is used to enrich total RNA samples for the small RNA fraction. It uses an efficient glass fiber filter based method. The mirVana™ miRNA Isolation Kit uses organic extraction followed by purification on a GFF under specialized binding and wash conditions. As a result, the kit effectively recovers all RNA – from large mRNA and ribosomal RNA down to 10-mers – from virtually all cell and tissue types. The

mirVana™ miRNA Isolation Kit enables the isolation of small RNA – containing total RNA from samples consisting of $10^2 – 10^7$ cultured cells. Total RNA isolation is done for CD34+ UCB cells and CD34$^+$ CD45$^+$ hematopoietic stem/progenitor cells from hESCs and iPSCs.

Quantification of total RNA isolated: Nanodrop 2000 is used to quantify total RNA obtained after isolation. A260/ A280 assesses the purity of the sample.

Culture of CD34$^+$ UCB cells: CD34$^+$ UCB cells were cultured in NuncΔ plastic tissue culture plates (Thermo Scientific) at 10^4 cells per well in a 12 well plate for derivation of erythroid cells. The culture media for growth and expansion contained DMEM High glucose, Hams F-12, 15% Human serum (Valley Biomedical), Pen/Strep, L-glutamine, 25µM β–ME, 5ng/mL sodium selenite, 50µM ethanolamine, 20mg/mL ascorbic acid (all from Invitrogen). In the first stage cells were cultured in the above defined medium for 8 days with 10^{-6} M hydrocortisone (Sigma), 100ng/mL SCF (Peprotech), 5ng/mL IL-3 (Peprotech), 3 IU/mL EPO (Fairview hospital pharmacy). One volume of cell culture was diluted in four volumes of fresh medium containing hydrocortisone, SCF, IL-3 and EPO. Cells were then collected by washing the wells properly with PBS. Centifugation at any stage is done at 3200rpm for 3 minutes. The cells are then collected in PBS and washed twice. Finally the cells are resuspended in the expansion media and reseeded at 5 x 10^4 cells/well onto MS-5 stromal layer. After 3 days cells are washed collected and put on adherent stromal layer without any exogenous growth factors. The cells are kept in this stage for 10 days.

MS-5 Stromal layer culture: Non-irradiated MS-5 stromal cells were prepared at a concentartion of 5 x 10^4 -20 x 10^4 cells/mL/well in 6-well tissue culture plate or in 12-well tissue culture plate 1-2 days prior to the seeding of CD34$^+$ cells. MS-5 are cultured in RPMI media supplemented with 10% FBS.

Flow cytometry analysis: Flow cytometry analysis is done for typical phenotypical markers during the time course of differentiation to erythroid cells. CD34, CD235a, CD45 APC antibodies (All antibodies used are from BD Biosciences).

Prepare cells to stain for Globin Expression: The cells at different time points

intracellularly for hemoglobins by using a protocol adapted from Thorpe et al.(47). Collect cells in FACS tubes in HBSS (Invitrogen). Pellet 5min, 1.2 krpm, r.t.; decant and re-suspend. Add 200µL Formalin (Electron Microscopy Sciences) and mix by hand. Incubate for about 20 minutes R.T. Pellet 3 min, 0.9 krpm, 0°C. Decant and transfer to ice. Quickly add 100µl 1:1 acetone:water pre-chilled to -20°C. Quickly cap, invert to mix, and incubate on ice 3min. Pellet 3min, 0.9 krpm, 0°C. Quickly decant and transfer to ice. Quickly add 100µl acetone pre-chilled to -20°C. Quickly cap, invert to mix, and incubate on ice 3 min. Pellet 3 min, 0.9 krpm, 0°C. Quickly add 100µl 1:1 acetone:water pre-chilled to -20°C. Quickly cap, invert to mix, and incubate on ice 3min. Pellet 3min, 0.9 krpm, 0°C. Quickly decant and transfer to ice. Re-suspend in HBSS supplemented with 2 % FBS.

Staining cells: Pellet 5min, 0.9 krpm, 4°C. Decant, ratchet and transfer to ice. For adult hemoglobin (Hb) add the antibody Hbß monoclonal antibody (SantaCruz). For fetal Hb add fetal Hemoglobin Mouse Anti-Human mAb, PE Conjugate (Invitrogen). Incubate on ice or fridge for 30-45 minutes. Fill tube with pre-chilled HBSS/ 2% FBS and invert to mix. Pellet 5min, 0.9 krpm, 4°C. Repeat the wash steps. Decant and transfer to ice (Keep in dark from this point). Add second (FITC) Ab for adult Hb and mix by hand. Incubate on ice or fridge in dark for 20 minutes. Wash twice as above. Re-suspend in 0.5-1mL pre-chilled HBSS / 2% FBS and store at 4°C in dark.

Globin expression analysis by quantitative polymerase chain reaction: Total RNA was extracted from cord blood cells put in erythroid conditions from different time points. This was done using mirVana™ miRNA Isolation Kit. The concentration was determined using Nanodrop. cDNA was extracted using NCode™ VILO™ miRNA cDNA Synthesis Kit (Invitrogen). The primers used were:

α-forward: CGGTCAACTTCAAGCTCCTAAG

α-reverse: CCGCCCACTCAGACTTTATT

β-forward: TACATTTGCTTCTGACACAAC

β-reverse: ACAGATCCCCAAAGGAC

γ- forward: CTTCAAGCTCCTGGGAAATGT

11

γ-reverse: GCAGAATAAAGCCTACCTTGAAAG

The primers are taken as described by EN. Olivier et al.(48).

Cytospin and Staining: For cytospin, cells are washed twice with FACS buffer and spinned in the cytospin apparatus. Slides are then stained with Wright-Giemsa reagents as described by Olivier et al.(48).

Erythroid specific differentiation from iPSCs: The cells were cultured on half density MEFs and differentiated by EB method as described above. These EBs were transferred at Day7 and Day11 to erythroid conditions. The protocol involves the following four steps:

Step1: The EBs were taken and put in Stage II media as described by the Elefanty group, supplemented with 10^{-6} M hydrocortisone (Sigma), 13ng/mL IL-3 (Peprotech), 13ng/mL BMP-4 (R & D systems), 33ng/mL Flt-3L (Peprotech), 100ng/mL SCF (Peprotech), and EPO (2.7U/mL) for 7 days.

Step2: The EBs were then kept in same platea and media was changed with Stage II media supplemented with 10^{-6} M hydrocortisone (Sigma), 13ng/mL IL-3 (Peprotech), 13ng/mL BMP-4 (R & D systems), 40ng/mL IGF-1 (Peprotech), 100ng/mL SCF (Peprotech), and EPO (2.7U/mL) for 7 days.

Step3: Cells were collected, washed with PBS, and seeded on confluent MS-5 stromal layer in StageII media supplemented with EPO (3U/mL) and 25µM hemin for 3 days. MS-5 was cultured as described above.

Step 4: Cells were collected and washed with PBS, and seeded on MS-5 for up to additional 7 days in Stage II media with 25µM hemin.

Hemin was prepared as described in Fibach et al.(49).

Flow Cytometry Analysis: Flow cytometry analysis is done for typical phenotypical markers during the time course of differentiation to erythroid cells. CD34, CD235a, CD45 APC antibodies (All from BD Biosciences) were used. IgG1 APC was used as Isotype control. All FACS analysis was done on FACS Calibur (BD Biosciences) and data analyzed by FlowJo software.

Isolation of CD34+ UCB cells

UCB serves as a positive control for the experiments as it has been used previously to derive erythroid cells by many groups(40). UCB blood is processed by the ficoll gradient centrifugation,which exploits differences in cell density to separate cells. The cells positive for the marker CD34 are isolated by column purification. Total volume of 120mL UCB, yielded $3.2x10^6$ cells, 95.9% pure population. Figure 4a shows the percentage of CD34+ cells present in the donor before the sort, labelled as the pre-sort fraction. The donor has 4.17% CD34+ cells pre-sort. On final purification through the column, the donor was found to have 95.9% CD34+cells. The yield and purity of the isolation is well optimized. To obtain high purity column purification is done twice. Figure 4b shows the number of CD34+cells lost during the two washes. The middle panel shows the percentage of CD34+ cells after the first column purification, which is about 50%. So column purification twice helps achieve high purity. The isolated CD34+ cells are used for obtaining the RNA as well as for generation of erythroid cells *in vitro*.

Differentiation of hESCs and iPSCs

Both stromal-based systems and stroma-free embryoid body (EB)-based systems for hematopoietic differentiation of hESCs and iPSCs. We use the "spin-EB" for hematopoietic differentiation of hESCs and iPSCs described by the Elefanty group(14). Compared to the stromal cell system, it helps in obtaining a much greater percentage of CD34+CD45+ cells: 20-30% in spin-EBs compared to 1-5 % in stromal cell system. The spin-EB system is not only more efficient, it is completely defined (stroma and serum-free) and allows precise quantification of hematopoietic cell development. Specifically, in Spin-EBs, 3000 undifferentiated hESCs or iPSCs are seeded per well of 96-well round bottom plates. To optimize the Spin EB protocol for getting a high yield of CD34+ CD45+ cells we tried changing the number of cells from 3000 cells/EB to 6000 cells/EB. It was found that 3000-4000 cells/EB works better in generating the required population. Different time-points were taken to analyze which day gives the highest yield, mostly Day11-13 was the time where the required population reached peak. A number of cell lines BC1iPSCs, Dub7 iPSCs and H9 hESCs were used to obtain the population. For all the cells lines, we kept few EBs in Stage I for the entire 13 days. Whereas other EBs were transferred to Stage II around Day 6-8, it was found that

Day6 seems to be the optimum time for transferring EBs to StageII. Now that we have optimized the yield of the required populations from these EBs, we plan to set up mass culture to extract the RNA. Figure 5 shows Day7 differentiation of Dub7 iPSC EBs.

Generation of erythroid cells from CD34$^+$ UCB cells:

All hematopoietic cell lineages initiate from a small population of HSCs according to pyramidal cellular hierarchy, that is, HSC, progenitors and mature cells. These HSCs express CD34 on their cell surface. Erythroid cells being mature cells can be generated *in vitro* from CD34$^+$ hematopoietic stem cells. Luc Douay and group describe an efficient method for generation of fully mature red blood cells from hematopoietic stem cells(45). The methodology is adapted for generation of erythroid cells from CD34$^+$ UCB cells. The method is a three step protocol: First, cell proliferation and erythroid differentiation are induced in presence of certain cytokines for 8 days. Second, cells are co-cultured with a stromal layer and erythropoietin alone for 3 days. Third, all exogenous factors are withdrawn and cells incubated on simple stroma for up to 10days. In the initial attempt to use the protocol BM-MSCs were used as stromal layer, and the culture medium was same as the one described by the group. The plates had floating brown mass in all the wells and no erythroid formation was seen. A possible explanation for the brown mass might be dead BM-MSCs or another possible reason might be the ferrous sulphate and ferric nitrate used in the media components. In the next attempt, MS-5 stomal layer was used and the model system was kept the same. The media used for growth and expansion of CB cells was the one described by our lab previously for lymphocyte development from hematopoietic stem cells(46). Efficient expansion of CD34$^+$ cord blood and their conversion to erythroid cells was observed. Figure 6a describes the model used for culturing the UCB CD34$^+$ cells. Day4 was typically the day cells were fed with fresh cytokines. Between Day11 and Day21 when all exogenous growth factors are removed and cells are only cultured on stromal layer, the cells moved towards erythroid lineage. One modification of this tried was to use EPO between Day11-21, which did not make much of a difference. So the model system used was the one described in Figure 6a. The cells showed a massive expansion in the culture media. The cells were seeded at 10^4 cells/well, an average of 27 fold expansion was seen by Day4, and 150 fold expansion by Day 7 (Figure 6b). When the cells were cultured in a 12 well plate, they seemed to be expanding better

14

than in a 6 well plate. This observation was true for multiple experiments, indicating the proximity of cells to be an important factor for efficient growth and expansion. The images shown in Figure 6c clearly indicate the expansion of cells in culture during the initial Days as well as on plating onto MS-5. After around Day 9 when the cells were spin, the pellet was red, as shown in Figure 6d. This red pellet indicates the formation of erythroid cells, which has to be confirmed by other methods. Figure 6e shows cytospin images for Day18 showing polychromatic erythroblast. Thus, we can conclude that cells are going towards the erythroid lineage but this has to be further tested.

Biomarker expression analysis of CD34$^+$ UCB cells in erythroid conditions

The surface markers are important to study the progression of CD34$^+$ UCB cells towards erythroid lineage. The markers expressed at different stages of the hematopoietic differentiation pathway are different. The progression of differentiation towards erythroid lineage in the proliferative phase system was documented by FACS analysis. The expression of pan leukocyte antigen CD45 and erythroid specific marker CD235a or glycophorin A was analyzed. For cells progressing towards erythroid lineage CD34 and CD45 expression is expected to be down-regulated, while CD235a is expected to be up-regulated. Figure 7a shows the FACS analysis for CD34, CD45, and CD235a for Day0, 6, 10 and 20. At Day0 the 96.3% cells are CD34$^+$, which decreases drastically by Day 20 where <0.5% population is CD34$^+$. Erythroid-specific marker CD235a increases from 5% to about 85% during the same course of days. This clearly indicates the progression towards erythroid lineage. Moreover, erthyroid cells have a CD45$^-$ phenotype as seen on Day 20, where < 2% cells show the marker CD45. At the end of the culture about 85% cells are CD235a positive and <2 % CD45 positive, indicating erythroid formation. Figure 7b summarizes the marker expression over the time couse of 20days in the model system. Day0, 6, 10 and 20 typically mark cells from each stage in the model i.e. before seeding the cells in erythroid conditions, before seeding the cells on a stromal layer, after seeding the cells on a stromal layer with exogenous factors and after seeding the cells on stromal layer without any exogenous factors respectively.

Hemoglobin switching

The switch of Hb production from predominantly fetal hemoglobin HbF ($\alpha_2\gamma_2$) in fetus to

15

exclusively adult hemoglobin HbA ($\alpha_2\text{ß}_2$) in the adult provides a model for the investigation of differentiation to mature erythroid cells. Culture conditions enhancing erythroid cell maturity, show a relative decrease in synthesis of fetal hemoglobin and an increase in synthesis of adult hemoglobin(50). The examination of HbA/HbF ratio shows the degree of maturity of terminally differentiated cells. There is a direct relationship between level of erythroid differentiation and HbA/HbF ratio. This ratio is expected to increase as the number of days in culture increase. To determine the type of hemoglobin, intracellular staining is done for three different time-points Day 0, 7 and 18. Figure 8a left panel represents the HbF. At Day 0 i.e. when the CD34^{+} UCB cells are not in erythroid conditions, the cells do not express any fetal hemoglobin. At Day 7 and 18 the cells express fetal hemoglobin, which is quiet contrary to expected. The Day 0 time-point analysis was done with both freshly isolated and thawed CD34^{+} UCB cells. Similar results were obtained with both fresh and frozen cells. The right panel Figure 8a represents the expression of adult hemoglobins. Hemoglobin is expressed at all three day time-points. Intracellular staining for hemoglobins was previously done by J Dias et al.(51). The information derived from the data obtained by their group was not sufficient enough to conclude hemoglobin switching. They observed the expression of fetal hemoglobins in the erythroid cells formed, contrary to expected. Figure 8b sums up the ratio of HbA/HbF which decreases as cells progress towards erythroid lineage. The information from intracellular hemoglobin switching is thus not sufficient to describe the hemoglobin switch. qPCR analysis as described in figure 8c shows absence of adult hemoglobin at Day 6 and 18, wheras a 28 fold increase in fetal hemoglobin. qPCR analysis data is insufficient to derve any conclusions about the hemoglobin switch. Other methods like hemoglobin electrophoresis or high performance liquid chromatography can be used to derive the necessary information.

Generation of Erythroid cells from Dub7 iPSCs

To generate erythroid cells from iPSCs the very first step was to induce hematopoietic differentiation, which was done using the Spin-EB method for differentiating enzyme adapted iPSCs. The cells were then subjected to erythroid conditions. We were not sure which day of transferring the EB to erythroid conditions would work better. So, we choose two time-points: one, was the Day 7 time-point where the EBs express CD34, CD43, CD41a which mark early populations of the hematopoietic hierarchy. Second,

the Day 11 EBs were picked as they express high levels of CD34 as well as CD45. CD45 more specifically marks the progenitor cells of the hematopoietic lineage. Figure 9a is the overall strategy of working on Dub7 iPSCs to generate erythroid cells. It illustrates the first step that is the formation of EBs from the Dub 7 iPSCs by using the Elefanty group protocol as described. EBs from Day 7 and Day 11 were then put in two different conditions for the generation of eryhroid cells. Condition 1 is a four step protocol adapted from E.N Olivier et al. as described in the materials and methods. The only difference was the use of StageII Spin-EB media instead of StemSpan media in the original protocol. It is hypothesized that the StageII media acts well to differentiate cells. Figure 9b illustrates the condition 1 that was used to culture the Dub7 EBs from Day 7 and Day 11. Figure 9c represents condition 2, which is essentially same as working with generation of erythroid cells from CD34$^+$ UCB cells.

Day 7 Dub7 iPSCs to erythroid conditions:

Till day 7 the Dub7 iPSC EBs are cultured in Stage I media with BMP-4, VEGF and SCF. Before putting the EBs in erythroid conditions a FACS analysis was done for CD34, CD235a and CD45. Figure 10a shows the Day 6 time-point of these EBs. As expected, the expression of CD45 is not evident, as CD45 marks for progenitor cells, which are not expected to be seen around Day 6 of differentiation. The cells express 12.4% CD34 as expected and 22.9% CD235a. The expression of CD235a in normal EB conditions means that the system has some erythroid inducing factors which have not been looked at before. Figure 10b is Day 12 time-point of Day7 Dub7 iPSC EBs put under condition 1. Day 12 would denote cells to be in the first stage of the four step protocol i.e. the EBs are cultured in presence of hydrocortisone, BMP-4, Flt-3L, SCF and EPO. The conditions are supposed to be more erythroid inducing but contrary to that, the expression of CD235a was down-regulated. CD34 and CD45 were both up-regulated. Figure 10c represents a Day 19 time-point where about 65% cells express CD45 indicating they are away from erythroid lineage. The cells at this stage are cultured in the presence of hydrocortisone, IL-3, BMP-4, SCF, EPO and IGF-1. In the first attempt when the cells were transferred to MS-5 with Hemin and EPO, brown floating clumps were seen which might be due to the hemin. Hemin has to be carefully made into a solution with neutral pH before working. Though in the second attempt the cells were put in a hemin solution of neutral pH, the progression towards erythroid

17

lineage was not observed. A few plates of EBs were supplied with the same batch of cytokines but no progression seen. The system has to be further optimized for obtaining erythroid cells from iPSCs. Figure 10d shows Day 7 and Day 19 images of Dub7 iPSC EBs. Day 7 image is the EB before putting in erythroid-specific conditions. Very few live cells could be obtained from the Day19 EB indicating death of cells.

Figure 11 represents the erythroid specific condition 2 for culturing the Dub7 iPSCs Day 7 EBs. Day 12 and Day 19 time-points as described by a and b were analyzed by FACS. CD45 expression was seen to decrease from 50 % to 2 % which is quiet optimal. Levels of CD34 and CD45 remained almost the same, with no significant increase or decrease, indicating these conditions are better optimized. We plan to work on these conditions with sorted CD34+ cells from Day 7 and a later day EBs to see if the same conditions work better. As the system has worked for generation of erythroid cells from CD34 expressing cord blood cells, it is expected to work for the CD34 expressing cells sorted from the embryoid bodies. Figure 11c represents images of Day 7 Dub7 iPSCs in conditions 2 on day 12 and day 19. By day 19, most cells were dead.

Day 11 Dub7 iPSCs in erythroid conditions:

Day 11 of EB differentiation represents a slightly later stage, where the hematopoietic progenitors start appearing. The Figure 12a and 12b represent Day11 Dub7 iPSC EBs in Stage I of conditions 1 and 2 respectively. In both the conditions the EBs are expressing a huge amount of CD45 which is not typical of an erythroid differentiation. The Figure 12c represents images of Day 11 Dub7 iPSC EBs in conditions 1 and 2 (left and right). When the EBs are dissociated and put in condition 2 they start forming a stromal layer like structure underneath. Though more optimization of the media and cytokines is required to obtain an efficient erythroid differentiation, we plan to put the EBs in same conditions by sorting $CD34^+$ $CD45^+$ cells from later day EB to see how they differentiate.

Discussion

The first component of study in the project is to profile the microRNAs from hESC and iPSC derived $CD34^+$ $CD45^+$ hematopoietic stem/progenitor cells and comparing them to $CD34^+$ UCB cells. This expression analysis along with the expression analysis of mRNAs might help elucidate the hematopoiesis pathway better. Most hematopoietic lineages have been derived from hESCs and iPSCs *in vitro* except for B lymphocytes and T lymphocytes. Understanding the hematopoietic pathway is an important part of understanding the derivation of these lymphocytes, as they arise from the common lymphoid progenitor as NK cells. NK cells have been successfully derived by our group(20). So, profiling the microRNA-mRNA expression might facilitate *in vitro* generation of B and T lymphocytes. In the long run, this might be helpful in treatment of blood diseases. We have optimized our embryoid body formation protocols to maximize the CD34+ CD45+ hematopoietic stem/ progenitor cells. Once we have a robust population of these cells, we plan to extract RNA by setting up a mass culture of each cell line. The RNA is then to be analyzed by nanostring technology. The data acquired can be studied using various bioinformatic tools like Pictar, TargetScan which analyze the microRNA-mRNA pathways. We expect to derive important data from the analysis.

The second component of study in the project is to profile the long non-coding RNAs from erythroid cells derived from hESCs and iPSCs. This will help understand the erythropoiesis pathway better. In the long run, altering the lncRNA expression such that it stimulates erythropoiesis might help generate red blood cells for transfusions. UCB cells are used as a control for the derivation of erythroid cells. We have derived erythroid cells *in vitro* from $CD34^+$ UCB cells. The cells form a red pellet on spinning and >85 % cells are expressing CD235a, indicating erythroid differentiation. However, to say that the cells have undergone terminal maturation to form the erythrocyte we still need to show hemoglobin switching. Study of Hb switching using intracellular FACS and qPCR still needs to be optimized. Other methods can be adopted if these do not work, like hemoglobin electrophoresis or high performance liquid chromatography. We are optimizing our protocols for the the generation of erythroid cells from iPSCs. We have tried two different protocols: One from Duoay et al. as described above (Condition1) where the cells do not seem to be progressing towards the erythroid lineage. The second one is the same as the one used for deriving erythroid cells from

19

UCB cells. The dissociated EBs form a stromal like structure which is not exactly known. In the initial attempts we have used unsorted EBs for the generation of erythroids. We plan to work with sorted cells in the future. As CD34$^+$ UCB cells, the sorted cells from hESCs and iPSCs might wrk in the same manner. If we successfully derive erythroid cells from these hESCs and iPSCs we plan to look for lncRNA expression. We are collaborating with Dr. Andrew Wilber at Southern Illinois university medical center to look at lncRNA expression in these cells. He plans to analyze the lncRNA expression from different time points along the development of erythroid cells.

Both studies will help understand the pathway of hematopoiesis and erythropoiesis better. If we learn about the key non-coding RNAs regulating the pathways, we can do *in-vivo* experiments to analyze the effect of over-expression of key RNAs. Studying the effect of modulation of level of expression of key non-coding RNAs might help in producing hematopoietic populations *in vitro* from hESCs and iPSCs. This might eventually be helpful for therapeutic purposes like in treatment of blood diseases.

Figure1:
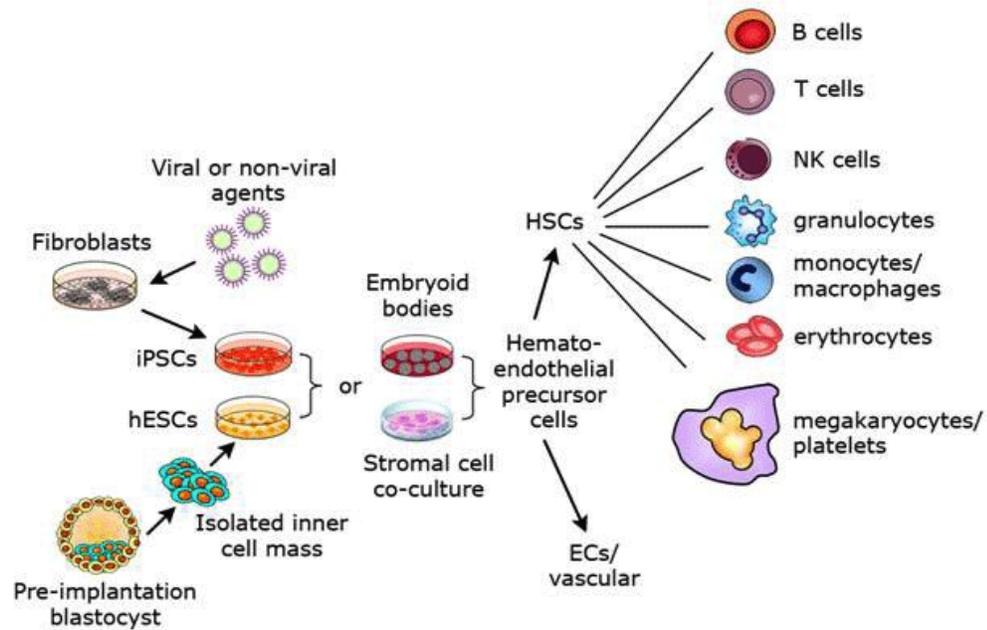
Figure1: **Derivation of hematopoietic and related cell lineages from human pluripotent stem cells**(2). HESCs and iPSCs can be differentiated *in vitro* by the embryoid body approach or by stromal cell co-culture to form HSCs. HSCs are cells in the bone marrow which replenish all blood cell types.
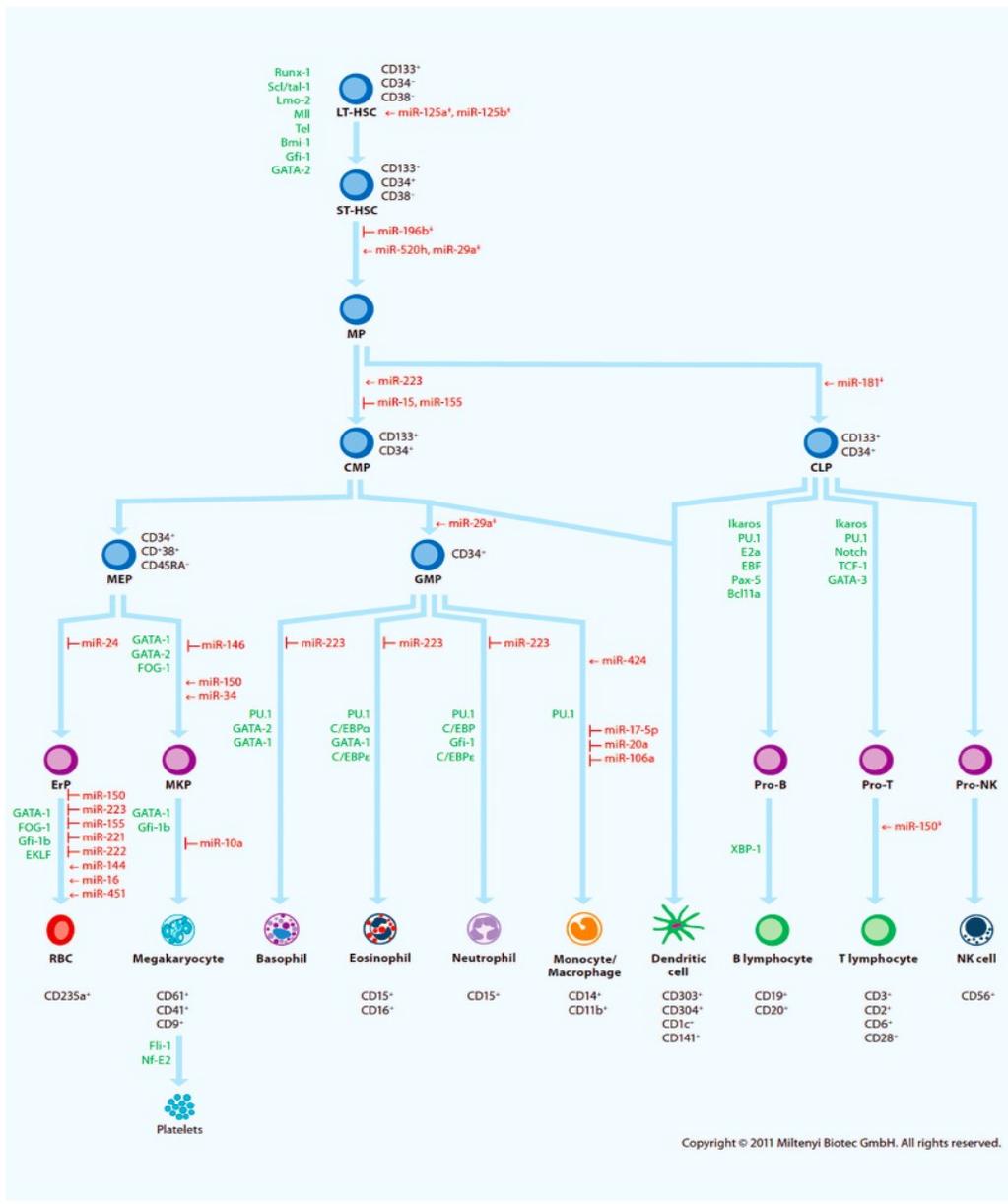
Figure2: **The role of microRNAs in hematopoietic development**(38). Various microRNAs are involved in different lineages of hematopoietic cells making the studies of microRNAs to be significant for understanding the pathway better.
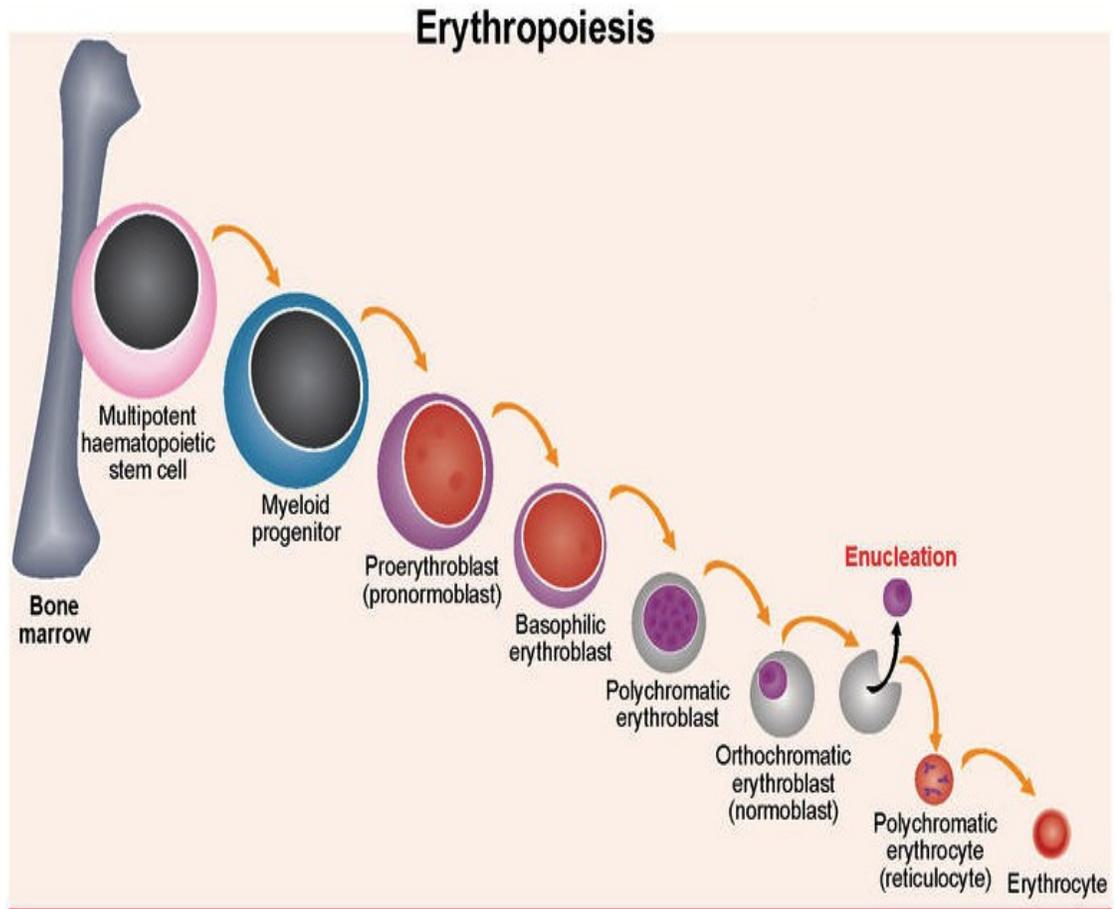
Figure 3: **Stages of erythroid differentiation**(52). The various stages of differentiation along the erythropoietic pathway, which represents enucleation towards the formation of erythrocyte. The various stages can be recapitulated *in vitro* for generating erythroid cells.

Figure 4

a)
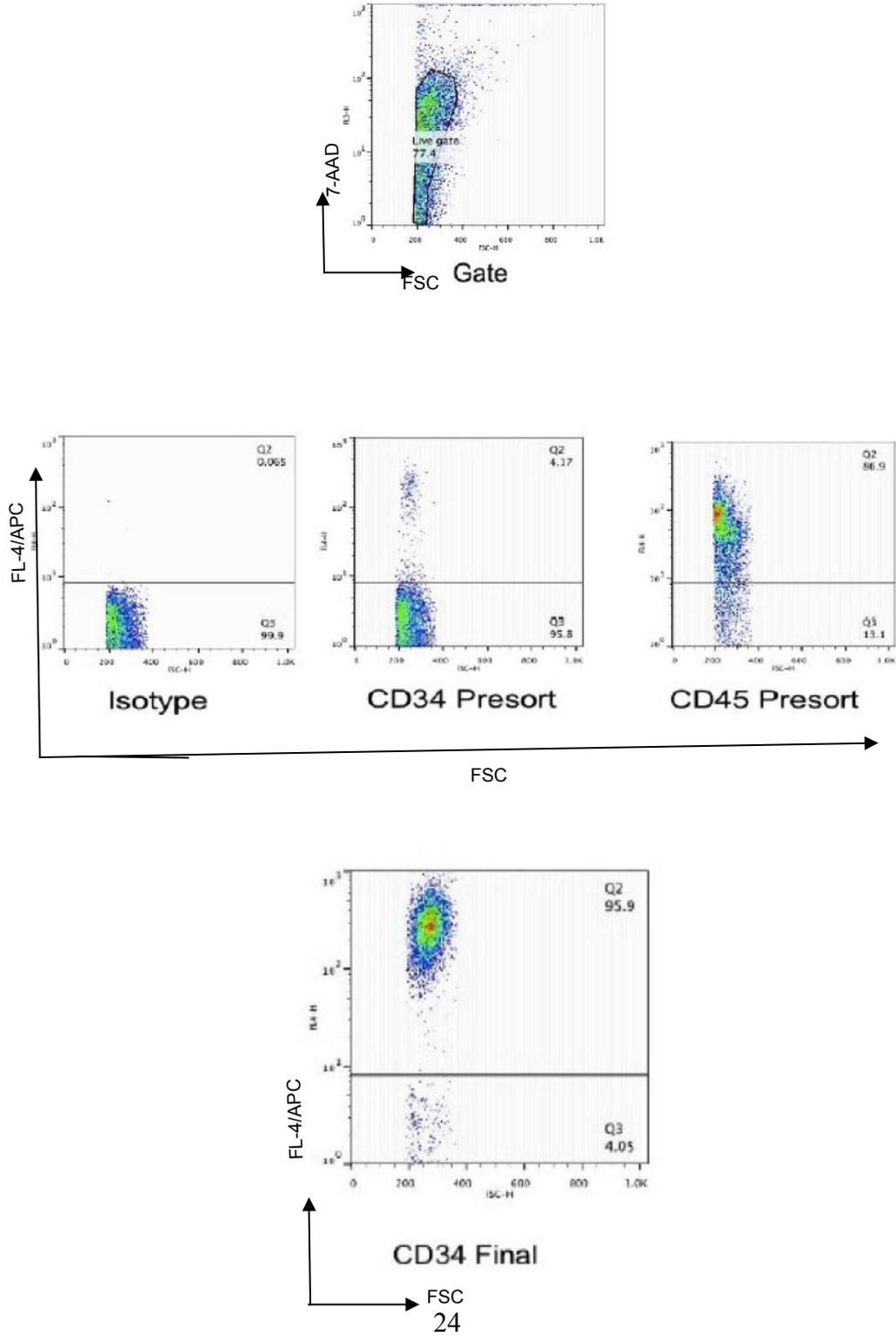
b)
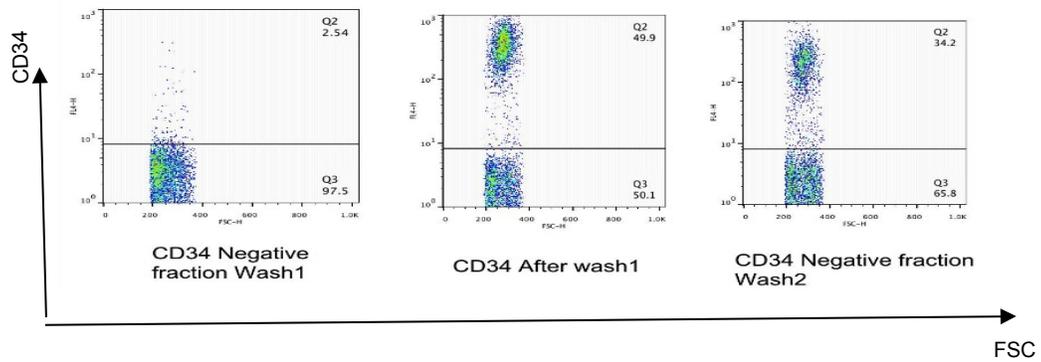
Figure 4: **Isolation of CD34+ UCB cells** UCB blood is processed by the ficoll gradient centrifugation,which exploits differences in cell density to separate cells. The cells positive for the marker CD34 are isolated by column purification a) Flow cytometry analysis for Isolation of CD34+ UCB cells showing the percentage of CD34+ cells before and after sorting I.e appox 5% and 96% repectively. b) Flow cytometry analysis of the negative fractions obtained on column purification, and the first wash indicating that column purification when done twice helps obtain higher purity of isolated cells.

Figure 5
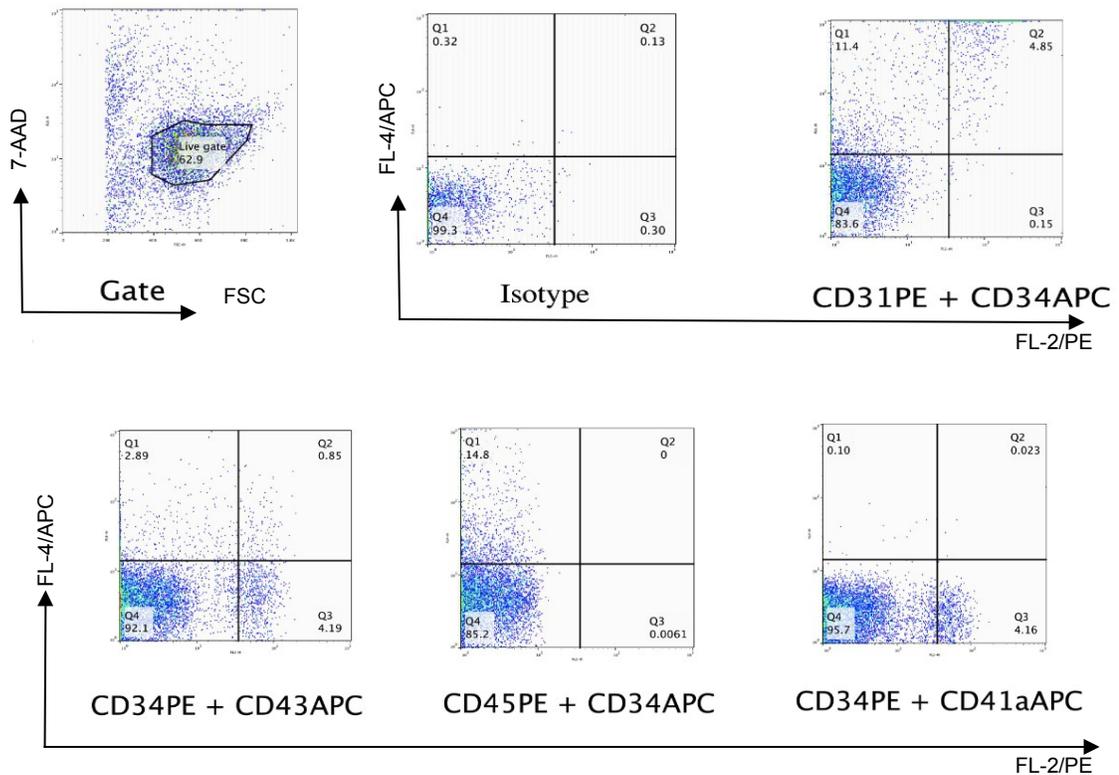
Figure 5: **Differentiation of hESCs and iPSCs** Day 7 Time-point of Dub7 iPSCs in Spin-EB differentiation. Shows a time-point in differentiation using the Spin-EB method, the CD34$^+$CD45$^+$
cells are not in required proportion till about Day 11-13 of differentiation. On Day7 cells are seen to be expressing CD34 but the amount of CD45 expressed is negligible.

Figure 6:

a)

b)

c)

Day 7

MS-5 stromal layer

Day 12

Day 17

Day 21
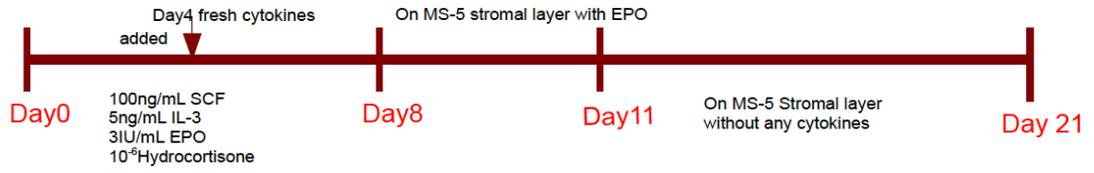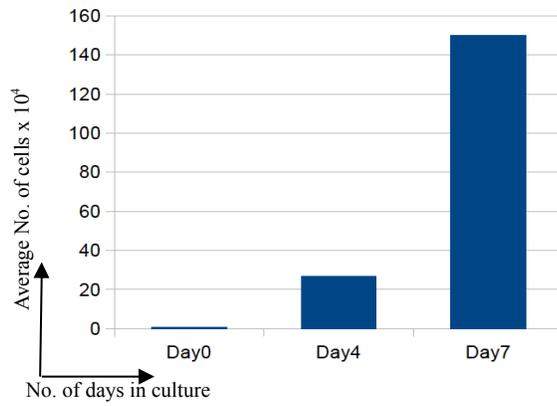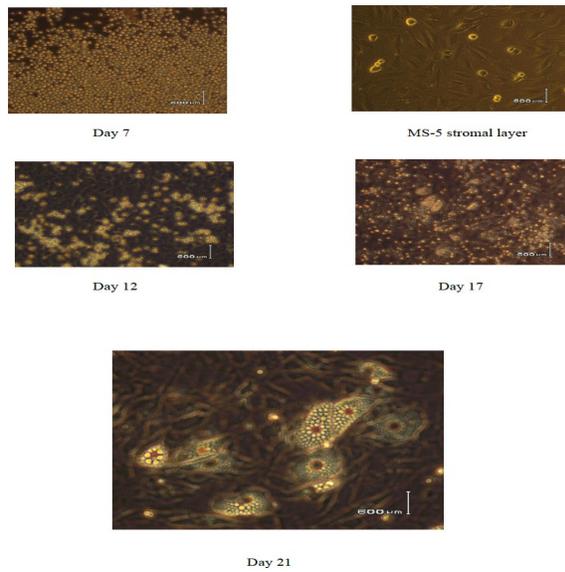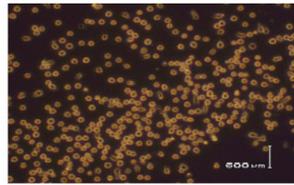
Day 0   Day 4

d)   e)
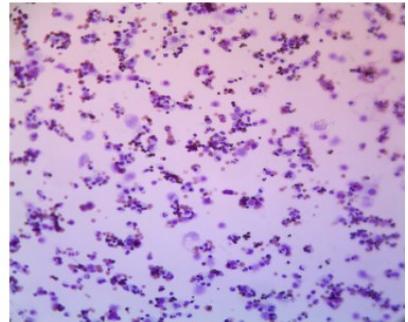
Figure 6: **Generation of erythroid cells from CD34$^+$ UCB cells** a) Model system used for the generation of erythroid cells from CD34$^+$ UCB cells is a 21 day culture system with three different stages as shown b) Haemocytometer count for the number of cells per well on Dy0,4 and 7 shows as number days in culture increases the number of cells increase too. Day 4 shows 27 fold increase in cell number, which increases to about 150 fold by Day 7. c) Pictures of UCB cells put in the model system for Day0, 4, 7, 12, 17, 21. MS-5 stromal layer is also shown. The fifure indicates increasing number of cells with increasing number of days in culture. The cells form typical colony like structures indicating some differentiation. d) Right tube represents a falcon with red pellet at Day 18 indicating the differentiation towards erythroid lineage. e) Cytospin picture at Day18 showing polychromatic erythroblast stage (20X).

Figure 7:

a)
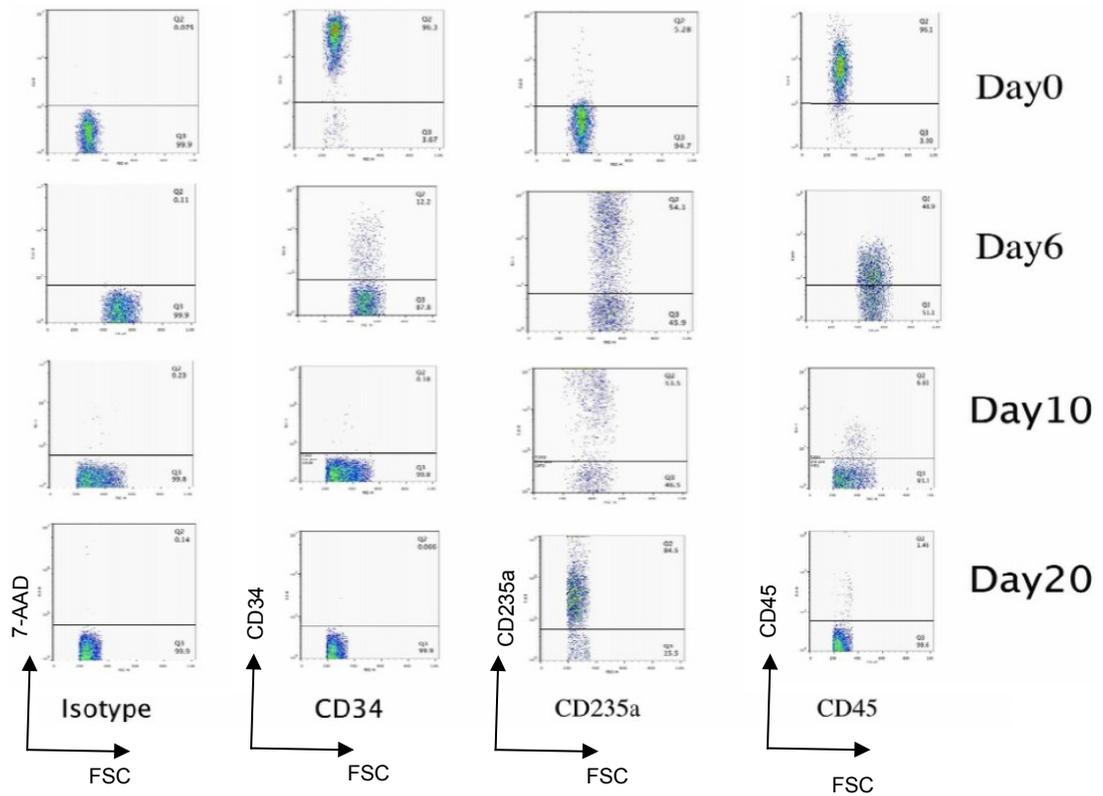
b)
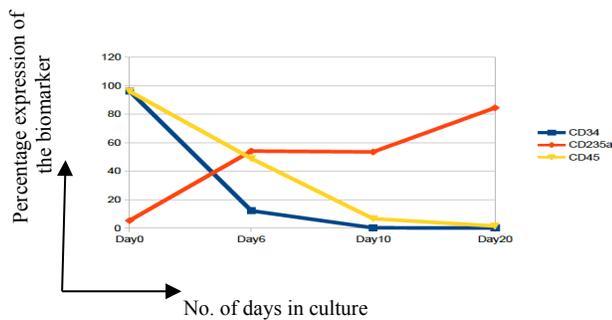
Figure 7: **Biomarker expression analysis of CD34^{+} UCB cells in erythroid conditions** a) Biomarker expression analysis for CD34^{+} UCB cells in erythroid conditions at Day 0, 6, 10 and 12. The glycophorin A marker increases as the cells progress towards erythroid lineage. The expression of CD34 and CD45 markers decreases. b) Graphical representation of the biomarker expression showing increase in gyycophorinA and decrease in CD34 and CD45 as number of days in culture increases.

29

Figure 8:

a)
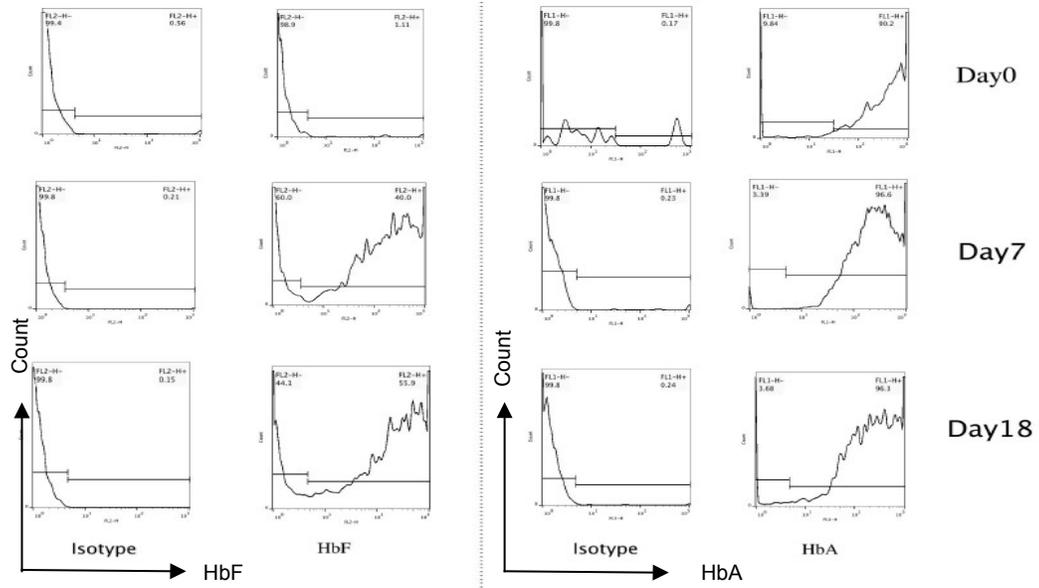
b)

	HbA/HbF Ratio
Day0	88.1
Day7	2.44
Day18	1.72

c)
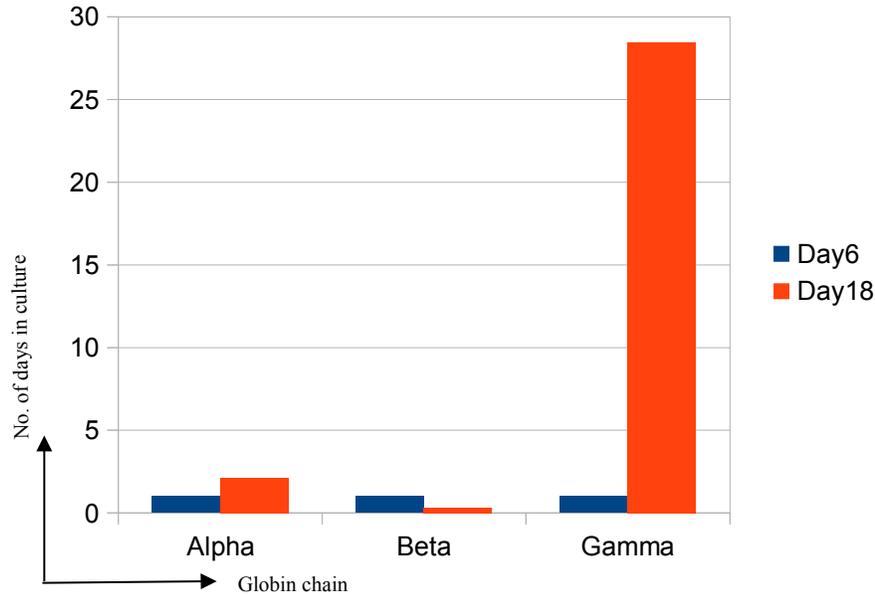
Figure 8: **Hemoglobin Switching** a) Hemoglobin expression analysis in CD34$^+$ UCB cells kept in erythroid conditions for Day 0, 7 and 18. Left panel represents fetal hemoglobin. Right panel represents adult hemoglobin. At Day 0 i.e. when the CD34$^+$ UCB cells are not in erythroid conditions, the cells do not express any fetal hemoglobin. At Day 7 and 18 the cells express fetal hemoglobin, which expected to increase as days in culture increase. b) HbA/HbF ratio at Day 0, 7 and 18 which is contrary to expected. c) qPCR analysis of Day 6 and Day18 in erythroid conditions. The expression of gamma chain increases, which signifies an increase in fetal hemoglobin as days in culture progres. X-axis: Three hemoglobin genes alpha, beta and gamma. Y-axis: Fold change normalized to GAPDH
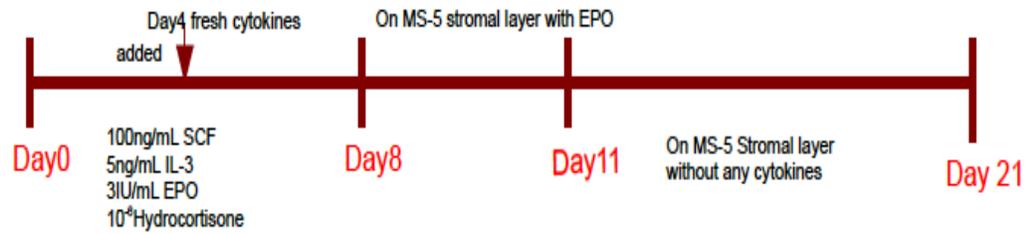
Figure 9:

a)
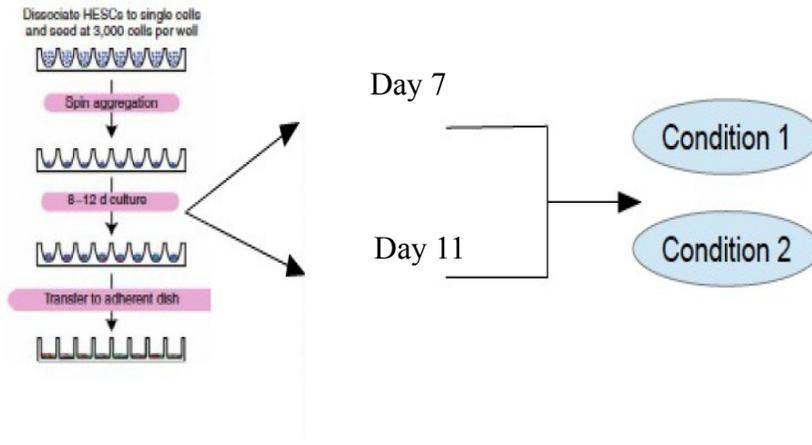
b)
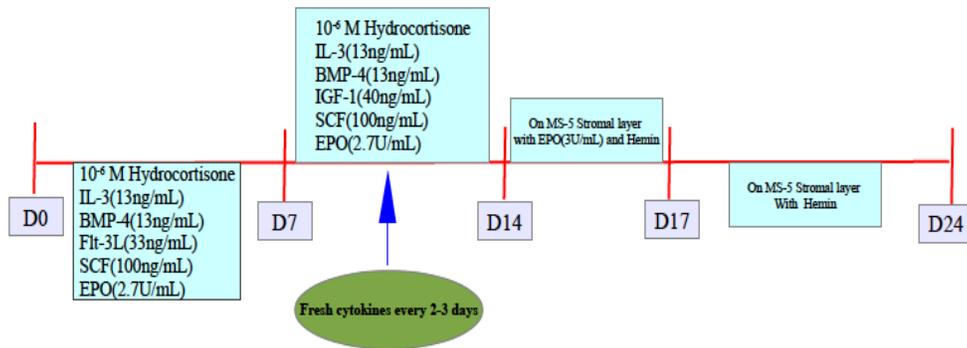
c)

Figure 9: **Models for Dub7 iPSC line in erythroid conditions**
a) Differentiation of hESCs and iPSCs by Spin-EB method and putting them in two different conditions which are discribbed in part b and c of the figure.
b) Condition1 of erythroid differentiation. Day 0 stands for the day of transfer. It is a 24 day cell culture system for generation of erythroid cells. The first two weeks comprise of changing the set of cytokines and last 10 days on MS-5 stromal layer making differentiation to erythroid cells complete.
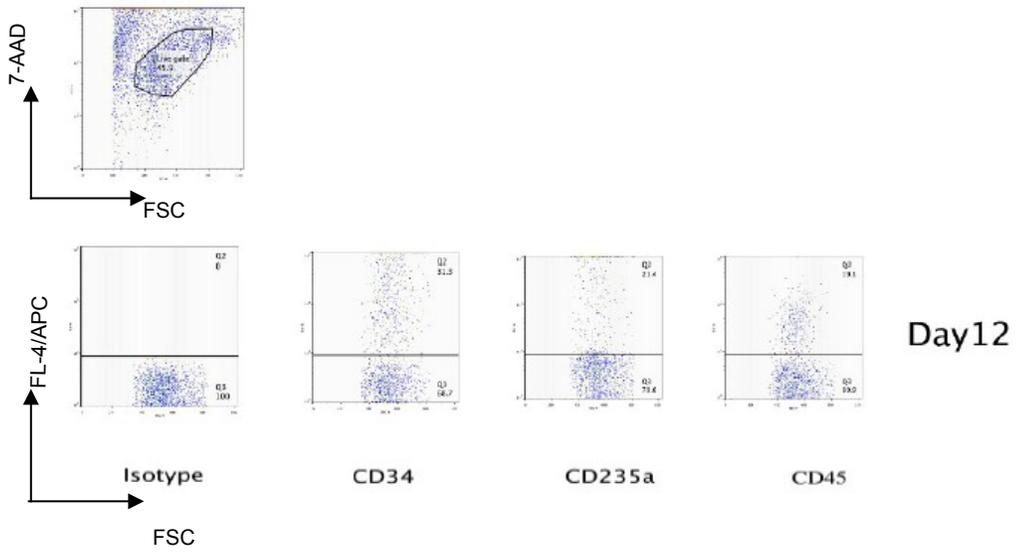c) Condition2 of erythroid differentiation. Day0 stands for the day of transfer. The condition is a 21 day culture system, same as the one used for differentiation of UCB to erythroid conditions.
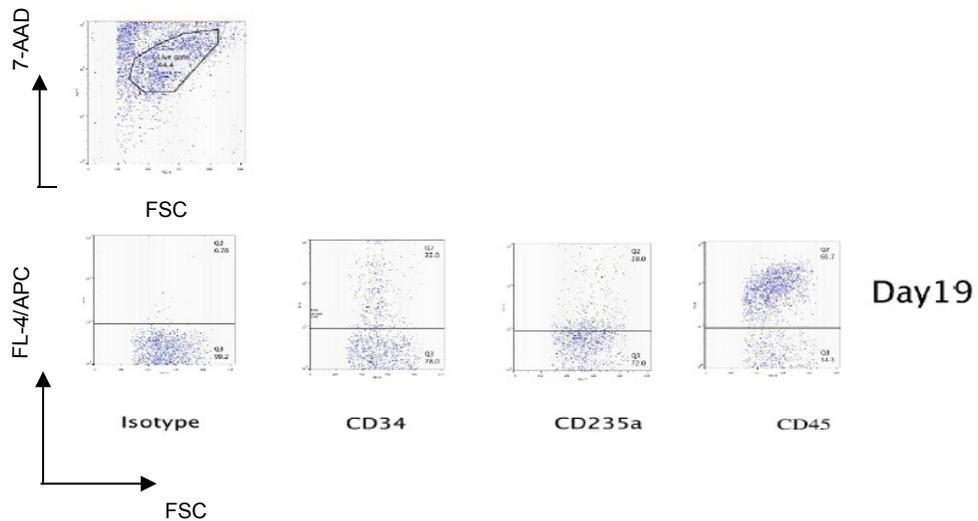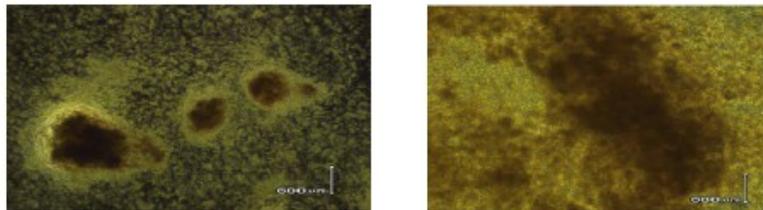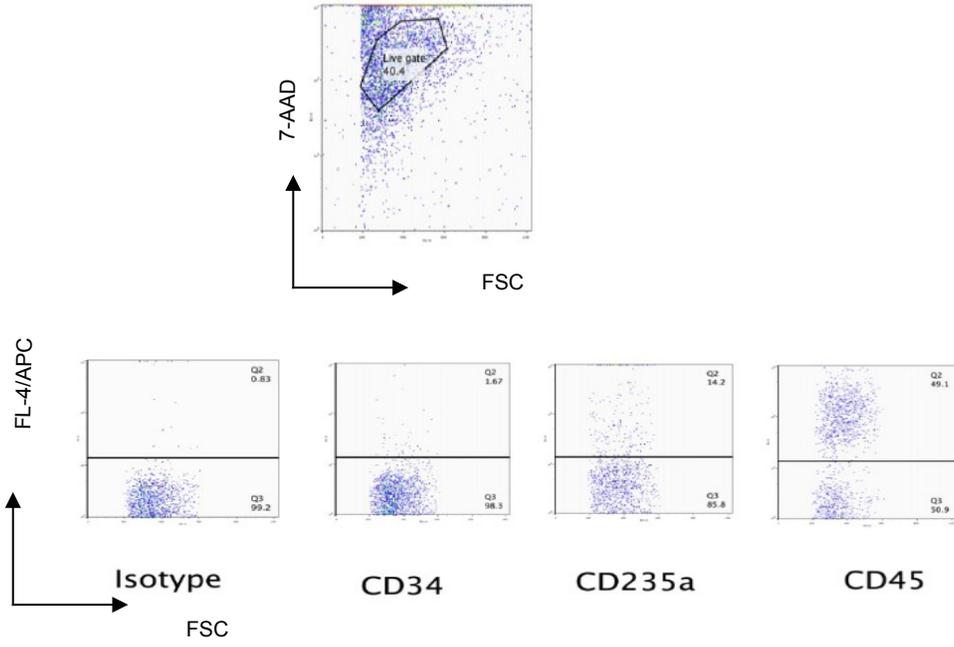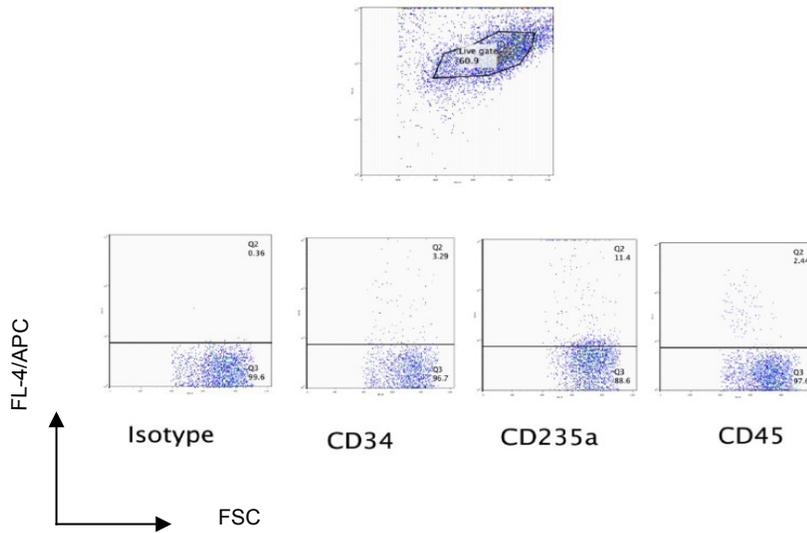
Figure 10:

a)

b)

34
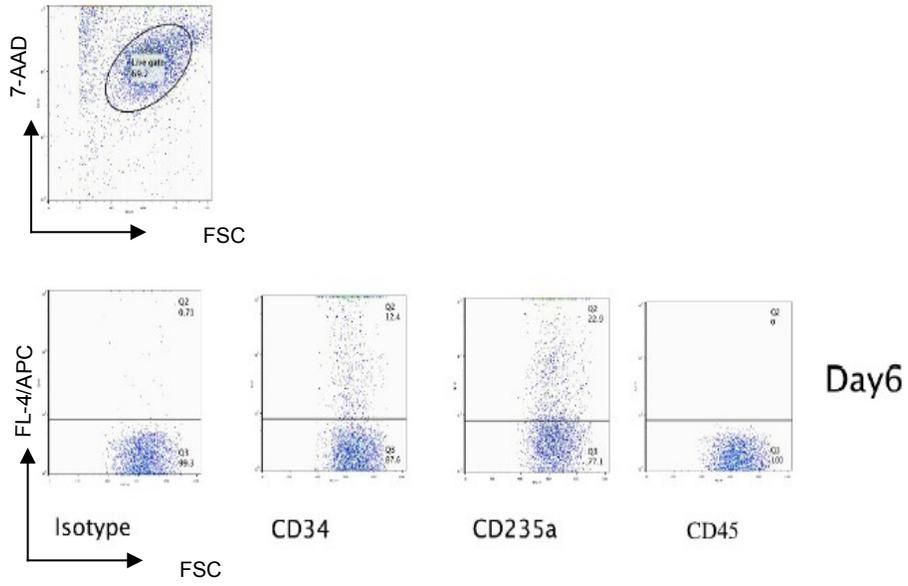
c)

d)

Figure 10: **Day 7 Dub7 iPSCs to erythroid conditions (Condition1)** a,b,c) Day 6, 12 and 19 FACS analysis for Dub7 iPSC EBs put in condition 1 described in Figure 6b. Figure 10a shows the Day 6 time-point of these EBs. As expected, the expression of CD45 is not evident, as CD45 marks for progenitor cells, which are not expected to be seen around Day 6 of differentiation. The cells express 12.4% CD34 as expected and 22.9% CD235a. In Figure 10b the conditions are supposed to be more erythroid inducing but contrary to that, the expression of CD235a was down-regulated. CD34 and CD45 were both up-regulated. Figure 10c represents a Day 19 time-point where about 65% cells express CD45 indicating they are away from erythroid lineage. d) Images of Day7 and Day19 EBs in condition1 described in figure 6b using phase contrast microscope. Day 7 image is the EB before putting in erythroid-specific conditions. Very few live cells could be obtained from the Day19 EB indicating death of cells.
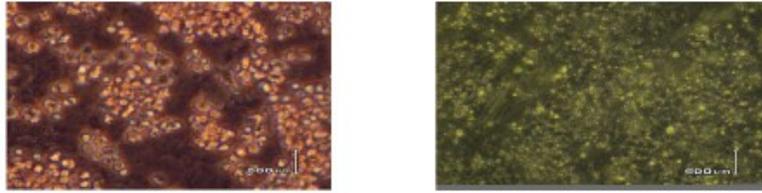
35

Figure 11:

a)

b)

36

c)

Figure 11: **Day 7 Dub7 iPSCs to erythroid conditions (Condition2)** a,b) Day 12 and Day 19 FACS analysis of Day 7 Dub7 iPSC EBs in erythroid conditions described in figure 6c. CD45 expression was seen to decrease from 50 % to 2 % which is quiet optimal. Levels of CD34 and CD45 remained almost the same, with no significant increase or decrease, indicating these conditions are better optimized. c) Images of Day 12 and Day 19 of Dub7 iPSC EBs in conditions described in figure 6c. By day 19, most cells were dead. The system needs further optimization.

Figure 12:

a)
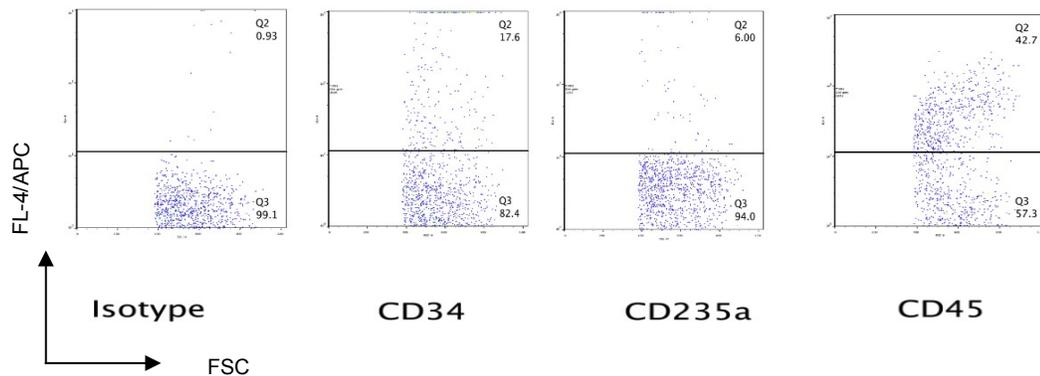
b)
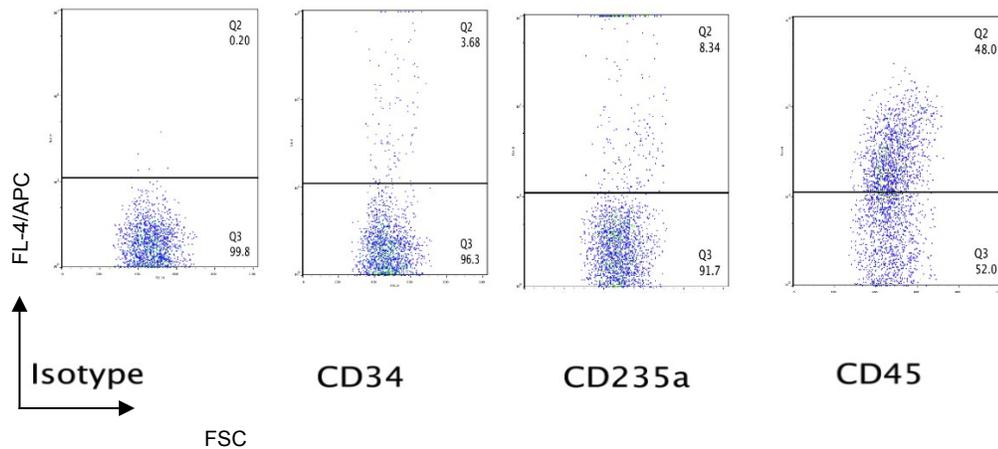
c)
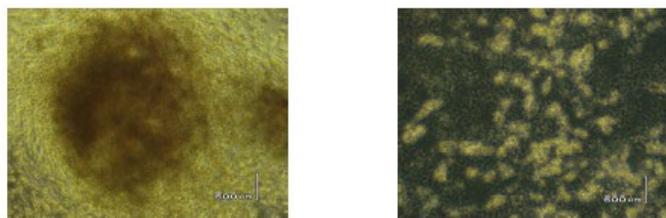
Figure 12: **Day 11 Dub7 iPSCs in erythroid conditions** a,b) FACS Analysis of D11 Dub7 iPSCEBs in erythroid derivation condition1 and 2 as described in figure 6b and 6c. The Figure 12a and 12b represent Day11 Dub7 iPSC EBs in Stage I of conditions 1 and 2 respectively. In both the conditions the EBs are expressing a huge amount of CD45 which is not typical of an erythroid differentiation. c) (Left) Image of Day 15 of D11 Dub7 iPSC EBs in condition1 of figure 6b (Right) Image of Day 10 of D11 Dub7 iPSC EBs in condition2 of figure 6c. When the EBs are dissociated and put in condition 2 they start forming a stromal layer like structure underneath.

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