Lineage Specific Reprogramming to Blood using a Cocktail of Transcription Factors

A Lineage Specific Reprogramming to Blood using a Cocktail of Transcription Factors

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DEDICATION

This work is dedicated to my loving husband, parents and in-laws without whose love and encouragement I would not have accomplished what I did.

ABSTRACT

A single fertilized cell has the ability to develop into any cell depending on the various cues it responds to. This ability to differentiate into desired cell types can be made use of in the field of developmental biology for studying early embryonic development and for regenerative medicine. Previous work in the lab showed that mouse ES cells engineered with an inducible construct co-expressing the hematopoietic regulatory factors SCL, LMO2 and GATA2 give very efficient hematopoiesis. In monolayer differentiation where hematopoiesis does not occur because the majority of cells differentiate towards ectoderm, expression of these 3 factors diverted cells towards hematopoietic lineage. My work in the lab addressed two questions: Can we optimize the system to obtain hematopoietic progenitors instead of differentiated blood cells? and How does the SCL complex reprogram cells at the molecular level? I added a cocktail of cytokines to the serum free, growth factor free medium to obtain undifferentiated hematopoietic progenitors. A short pulse of induction was sufficient to obtain large number of CD41+, hemoglobin expressing, round semi adherent cells. This treatment gave rise to progenitors of myeloid, erythroid and megakaryocytic lineages proving the multipotent nature of the blood cells that differentiated to both hematopoietic progenitors and committed erythroid cells. To understand the reprogramming potential of the SCL complex at the transcriptional level I performed two RNA sequencing experiments. The first experiment evaluated the early changes and showed that the SCL complex up-regulated many important hematopoietic genes including SCL, LMO2, GATA2, Lyl1 and Gfi1 within 6 hours, but other genes (globins) required a longer period of induction. The second experiment evaluated cells 3 days after a reprogramming pulse. The data showed that the non-reprogrammed (CD41-) cells expressed hematopoietic genes, but at lower levels compared to the fully reprogrammed cells (CD41+) indicating that the

cells were not completely reprogrammed. Surprisingly the CD41- cells had higher expression of endodermal genes indicating that the cell could have reverted endoderm, a nearby lineage during the chase period. The data also showed a decrease in the ectodermal genes in both CD41+ and CD41- cells suggesting that the triple construct may be stably erasing the ectodermal program even in non-reprogrammed cells. These results improved the efficiency of the system and shed light on the mechanism of the SCL-LMO2-GATA2 action in lineage specific reprogramming.

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INTRODUCTION

Development occurs through stepwise commitment of undifferentiated cells towards more restricted lineages [1, 2]. How cells change their lineage and become committed to a particular fate is a challenging topic. Molecular biological advancement and genetic manipulations have made it possible to identify the role of genes and how their involvement alters cell fate. Early mouse embryo development gives rise to three primary germ layers: ectoderm, mesoderm and endoderm. During the process of gastrulation, epiblast cells pass through the primitive streak giving rise to mesoderm and endoderm and the cells which do not cross the primitive streak becomes the ectoderm [2]. How epiblast cells migrate and what causes the induction of different germ layers is an area of active research. The location of the precursors and the different signals the cells respond to play key roles in this process. Using gene targeting and gene expression assays it has been shown that various signal molecules like BMP4, Wnt, TGFβ and Activin are all involved in early developmental processes. Transcription factors (TF) also play an important role in specification and determination of cells by responding to biological signals and controlling the transcriptional rates of genes [3, 7].

In a recent study Ismailoglu engineered mouse embryonic stem cells (mES) with inducible expression of blood-patterning transcription factors SCL/Tal1, LMO2, and GATA2 and were able to reprogram somatic cells derived from pluripotent cells into blood cells efficiently in monolayer culture [5].

My work was aimed towards understanding this lineage specific reprogramming process by studying the functional characteristics of the blood cells obtained from

ES cells. I addressed the following questions. What types of cells are being reprogrammed? Do the reprogrammed cells have hematopoietic progenitor cell characteristics? What happens to the non-reprogrammed cells? How efficient is the inducible system for hematopoietic differentiation? Is the transcription factor cocktail sufficient to reprogram even somatic cells of other germ layers to blood cells?

Embryonic Stem Cells

Embryonic stem (ES) cells were first derived by Evans and Kaufman in 1981, from the inner cell mass of the blastocyst embryo [65, 70]. ES cells can self renew and can differentiate into any somatic cell type and the germ lineage [7, 8]. Because of the pluripotency of ES cells they have tremendous potential in the field of regenerative medicine [6, 8]. Current research is focused on understanding the molecular mechanisms involved in ES cell differentiation towards a particular cell lineage in order to make it more efficient. Due to ethical issues related to the use of human embryos, limited accesses and the small size of other vertebrate embryo, ES cells have become an excellent tool in the field of developmental biology to study molecular mechanisms and cell differentiation processes. Mouse ES cells have been used extensively because of the ability to differentiate into a broad spectrum of lineages. They provide an unlimited source of cells for conducting research both *in-vivo* and *in-vitro* providing a model of early development. ES cells are maintained in an undifferentiated state by co-culturing with mouse embryonic fibroblasts cell (Mefs). These feeder cells secrete a molecule called leukemia inhibitory factor (LIF) [66], which functions through activating the STAT3 pathway to help in maintain the undifferentiated

state of ES cells [9]. Molecular analysis studies have now shown that other molecules like BMP4 play similar roles like LIF. Once the feeder layer is removed ES cells will differentiate into different lineages under appropriate conditions. Many protocols have been developed for lineage specific differentiation of mES cells. Lineage specific reprogramming using TF was established with studies using myogenic transcription factors MyoD, over expression of which converted fibroblasts to myoblasts [62, 63]. For lineage specific differentiation of ES cells it is important to develop a protocol which gives efficient and reproducible numbers of desired cell types. The desired end population should be pure without unwanted or intermediate cells. Lastly the developed cells should be functionally similar to wild type cells in culture and retain these characteristics even after transplanting into model animals. It has been a major challenge to successfully transplant ES derived cells in animal models and have them maintain their characteristics after transplantation [7, 10,19].

Hematopoiesis:

Mesoderm gives rise to different lineages including hematopoietic, vascular and cardiac. Hematopoietic development can easily be studied using ES cells culture and is one of the best characterized lineages [11]. Hematopoiesis is a complex multistep process which refers to the formation and development of blood cells. During development hematopoiesis occurs in sequential processes first as primitive hematopoiesis in blood island and yolk sac and produce red blood cells expressing embryonic globins.

Later it occurs as definitive hematopoiesis in the fetal liver giving rise to red blood cells expressing fetal hemoglobin [12]. Much progress has been made in understanding the genes involved in the early stage of hematopoietic development. With the help of modern molecular biology and genetic tools like targeted gene deletion it has been possible to understand the function of an individual gene or its products during development [13].

The hematopoietic system is a diverse, multi-lineage system which undergoes changes throughout the development. It begins with simple, limited number of specialized lineages in the yolk sac which then matures with development into a complex multiple lineage system. One model postulated that blood and blood vessels are formed from the “hemangioblast” which is derived from mesodermal cells and is a common precursor for hematopoietic and endothelial cells [14, 67]. Yolk sac blood islands consist of primitive erythrocytes surrounded by endothelial cells [15]. Primitive erythrocytes are very different from fetal or adult erythrocytes and their production is called primitive erythropoiesis. Definitive hematopoiesis consists of development of other lineages including lymphoid lineages.

The hematopoietic system has a hierarchical organization in which hematopoietic stem cells lose their self renewal potential and give rise to progenitors of different hematopoietic lineages [16, 18]. These progenitors finally give rise to a mature population of differentiated cells. For maintaining the self renewal potential of hematopoietic stem cells (HSC) which are required for life long hematopoiesis there are factors like SCF/c-Kit receptor, Notch, wingless-type (Wnt), sonic-hedgehog (shh) and Smad (families of TGF-β, activins and bone morphogenic proteins (BMP4) signaling pathways) which support the expansion of HSC pool

[19]. Studies have shown that Activin A/Nodal, Bmp4 and FGF are important for mesodermal patterning and Wnt plays a crucial role in formation of mesodermal layer and patterning. Wnt also modulates the fate of HSC by regulating the bone marrow niche [20]. Studies have demonstrated that Wnt is required for maintaining and proliferating HSC as it up regulates the expression of HoxB4 and Notch genes which are required for HSC self renewal [69].

Use of serum in the medium is an effective and efficient way of differentiating ES cells towards the hematopoietic lineage. Using gene expression assays, identification of surface markers and appearance of progenitor cells demonstrates hematopoietic commitment of ES cells in culture [21]. These cultures also express Flk-1 which is a hematopoeitc/vascular tyrosine kinase receptor. Keller et al [22] have shown that culturing ES cells as embryoid bodies (EBs) under appropriate conditions gives rise to primitive erythroid cells which are followed by development of macrophage and mast cells.

Gene targeting experiments have demonstrated that specific transcription factors like SCL/Tal1, RUNX1, GATA1 are required at specific stages of hematopoietic development. Primitive and definitive erythroid lineages require SCL making it an essential factor for development of all hematopoietic lineages. RUNX1 is required for definitive hematopoiesis and GATA1 is required during late primitive and definitive erythroid stages [23]. Also, ES culture system has made it possible to identify the molecular targets of a gene of interest by analyzing mutations in EB's. One of the major objectives in this field of research is development of HSC's which can be transplanted in to hematopoietic deficient animals to treat blood related disorders. For this objective to be successful it is

important that stem cells give rise to multi-lineage hematopoietic populations, capable of long term survival and also home to bone marrow after transplantation.

There have been many attempts to generate HSC from ES cells *in-vitro*. Kyba et.al [19] transplanted ES derived HSC with inducible HoxB4 expression, which were evident even after 12 weeks of transplantation. However the repopulated population had more myeloid cells compared to lymphoid cells suggesting that HoxB4 may skew differentiation. In another experiment CD45+/c-Kit+ EB's were transplanted into irradiated animals which were capable of giving rise to both myeloid and lymphoid lineages [25]. But this study was conducted using medium with serum and presence of unknown factors in serum could promote multiple lineage differentiation and repopulation of HSC. It is essential to reproduce similar results in serum free defined medium to prove that HSC can be developed *in vitro* using ES cell culture model.

Further studies are necessary to determine if these HSC are comparable to HSC in fetal liver and adult bone marrow. It has been demonstrated that by using ES cells it is possible to understand initial stages of hematopoiesis and now using the same system we can study the regulation of hematopoietic commitment. Different groups have used serum free medium and demonstrated that factors like BMP4 and VEGF act during specific stages of hematopoietic development [26]. BMP4 induces a Flk-1+ population and VEGF promotes SCL/Tal-1 expressing hematopoietic and vascular progenitors within the Flk-1+ population.

Gene knockout experiments in mice have provided evidence for many transcription factors that are required for hematopoiesis. Analysis of knockouts has helped to understand the molecular and cellular functions of these transcription factors. Also chemical and proteomic analyses have provided insights about the protein interactions and signaling pathways that regulate the activities of factors required during hematopoietic development [27].

SCL/ Tal1:

SCL/Tal1 is a basic helix loop helix protein and was first discovered as T-cell oncogene [28]. Targeted gene ablation techniques in zebra fish have revealed SCL as an important transcription factor in hematopoietic stem cell differentiation to specify all blood lineages [29]. SCL/Tal-1 binds to a consensus DNA motif called the E-box.

This gene is considered to be the master regulator of hematopoiesis. Studies have indicated its expression in endothelial and neural progenitor cells but the role in these tissues remains unknown. SCL-/- knockout mice die at embryonic day 8 - 8.5 due to sever anemia [30] however ectopic expression of SCL in the zebra fish mutant *cloche* rescued blood and endothelial defects suggesting a role for SCL in self renewal and proliferation of multipotent hematopoietic cells and as a master regulator of erythroid differentiation [31]. Reports have indicated that SCL is expressed in AGM, liver and yolk sac during mouse development [62]. Studies conducted in zebra fish embryos by Green et.al. [32] showed SCL to be co-expressed in a common progenitor for blood and endothelium in the sixth somite stage embryo. These SCL+/Flk+ cells were followed by SCL+/Flk- hematopoietic and SCL-/Flk+ endothelial cells in the tenth somite stage embryo.

LMO2:

LMO2 contains two cystine rich regions called the LIM domains [3]. It was first identified by chromosomal translocations in activated T cell leukemia [34]. Studies have shown that LMO2 null mice die at E9-10 due lack of erythropoiesis [35]. Similar experiment with homozygous mutant LMO2-/- mouse embryonic stem cells showed an absence of hematopoiesis. However over expression of LMO2 rescued the phenotype. This disruption of hematopoiesis is thought to be due to failure of interaction of LMO2 with SCL and the DNA binding protein GATA1. The data also strongly supported that LMO2 is the bridging molecule linking different DNA binding factors like SCL, GATA1, and E47 which form a complex in erythroid cells indicating that LMO2 is an important factor for hematopoiesis [36]. LMO2 has also been shown to interact with GATA2 which is expressed very early in hematopoiesis before GATA1 [37].

GATA2:

GATA factors consist of two zinc finger domains which bind to a A/TGATAA/G consensus DNA binding motif [38]. GATA1 is expressed in definitive hematopoietic cells like mast cells, erythroid cells, eosinophil and dentritic cells. GATA1 [39] has been shown to interact with protein and co factors of many of the transcription factors involved in hematopoiesis including SCL/Tal-1. GATA2 is a related zinc finger protein and is an important factor in maintaining the early hematopoietic cells. GATA3 is expressed in T cells [40]. Previous gene targeting studies have shown that GATA2-/- mice die early at E10-11 with sever anemia. GATA2 null ES cells show reduced expression of c-Kit and stem cell factor. These data provide evidence that GATA2 is necessary for maintaining

and proliferating early hematopoietic progenitor as well as megakaryocytes and mast cell lineages [41]. This study also indicated that GATA2 is required for early erythropoiesis but not necessary for maturation of erythroid cells and macrophages.

SCL/Tal-1/LMO2/GATA-1Ldb-1/E2A Complex:

SCL/Tal-1 forms a multimeric complex with the ubiquitous bHLH factor E2A, hematopoietic LMO2 and Ldb-1 Lim-domain binding transcription factor and hematopoietic LMO2 with GATA-1 proteins forming a pentameric complex that binds to the E-box/GATA DNA motifs [43]. SCL/Tal-1 mirrors the expression of GATA-1 as the SCL/Tal-1 knockouts results total absence of hematopoeisis. SCL/Tal-1 is critical for erythroid differentiation as conditional knockouts of SCL/Tal-1 during hematopoeisis result in no erythropoeisis. LMO2 -/- null mutants behaves similar to SCL -/- mutants as they die in the embryonic stage due to sever anemia [44]. These results suggest that LMO2 acts as a bridge between SCL/Tal-1 and GATA-1 in the complex. This result was further demonstrated by co-expressing LMO2/SCL/Tal-1/GATA-1 in Xenopus embryos which promoted erythropoiesis. In contrast, knocking down the expression of Ldb-1 using antisense morpholino RNA resulted in defective hematopoiesis. Recent studies have demonstrated that the complex also interacts with the ETO-2 repressor protein. ETO-2 is shown to be essential for definitive erythropoiesis by the knockdown experiments conducted in zebra fish embryos [45]. It is still not clear how the ETO-2 repressor protein functions with SCL/Tal-1 during early erythropoiesis.

Earlier studies on mesodermal patterning of SCL by Ismailoglu et al [48] used an iSCL cell line which expresses SCL under the influence of doxycyline and showed that SCL acts as a master regulator capable of reprogramming lineages like cardiac or paraxial mesoderm into hematopoietic mesoderm. This observation was demonstrated by use of Noggin, a BMP4 antagonist [49] which increases the paraxial mesoderm differentiation and with inducible expression of SCL this effect was reversed resulting in increase of hematopoietic mesoderm.

A pulse-chase experiment using iSCL EB showed that the effect of SCL was strong at day 3-4, a time in which Brachyury, a maker of early mesoderm is expressed. With SCL expression there was an increase in hematopoietic colonies. The results also suggested cell autonomous behavior of SCL and showed that only the cells expressing SCL had enhanced hematopoiesis. Previous studies involving hematopoietic differentiation have shown that SCL interacts with many other factors which could be involved early in hematopoiesis like LMO2, GATA1 and GATA2 [45, 50]. Based on this knowledge further experiment was conducted by generating iSCL-LMO2-GATA2 inducible mouse ES cells and studying their ability to drive hematopoiesis.

Ismailoglu[5] demonstrated that co-expression of SCL along with LMO2 and GATA2 in a mouse ES cell line with a conditional doxycycline inducible expression is able to promote hematopoietic cells at the expense of other lineages like cardiac mesoderm and paraxial mesoderm. For this study they used GATA-2 instead of GATA-1 because GATA-2 is expressed much earlier in hematopoiesis compared to GATA-1 which is required mainly for erythroid differentiation. To obtain a population of mixed lineages GATA-2 was used. Also,

biochemical studies revealed the association of GATA2 in a complex along with SCL/Tal-1 and LMO2. GATA2 mimics the phenotype of SCL-/- and LMO2-/- embryos showing anemia and embryonic death at E10 [46] suggesting that a SCL, LMO2, GATA2 complex is required for embryonic hematopoiesis. These factors interact together as a multimeric complex during erythrocytic or megakaryocytic differentiation but, their interaction has not been proven in early embryogenesis.

Serum free monolayer culture of mES cells.

Differentiation of ES cells into specific lineages is usually derived through EB differentiation [47]. EB's are aggregates of cells which form a three dimensional structure mimicking the early embryo development by cell -cell interaction and forming a number of embryonic lineages. The EBs are usually cultured in media with serum to provide necessary growth factors for development [47, 2].

Due to the presence of unknown factors in the serum, batch to batch variation of serum and also because of the complex structure of EB's it is difficult to understand the effect and function of an individual factor [5]. There are other simpler ways of differentiation like monolayer differentiation and cells will differentiate in serum free medium which can be useful to understand the effects of single factors. Due to its scalability and reduced variability compared to EBs monolayer differentiation is advantageous.

Hematopoiesis requires an interaction between nascent mesoderm and primitive or definitive endoderm in a 3D structure [48], so hematopoiesis does not occur in a serum free monolayer differentiation. Ismailoglu [5] obtained hematopoietic

cells from mouse ES cells cultured in serum free monolayer culture by co-expressing SCL, LMO2, GATA2 showing that these factors can convert non-hematopoietic cell to blood cells at high efficiency.

iSCL-LMO2-GATA2 cocktail interaction induces hematopoiesis in serum-free monolayer culture:

Ismailoglu et. al. [5] used a tricistronic construct consisting of SCL-iresLMO2-ires-GATA2 transcription factors. They used mouse ES cell to target this construct such that these three factors were induced conditionally by doxycycline. The investigators observed an increase of hematopoietic cells after 4 days of Doxycycline induction. Around 70% of cells were marked by the expression of hematopoietic marker CD41, and morphologically cells were rounded and lifted off the plate. This data was further confirmed by very high expression of globin genes. Expression of embryonic globins equaled the levels of GAPDH [51]. In contrast, the expression of neuroectodermal, endodermal, and non-hematopoietic genes were suppressed with increased hematopoietic gene expression.

One striking observation was the cells did not express mesoendodermal (PDGFRα) [52] and early mesodermal (Flk1) [53] markers before the appearance of hematopoiesis demonstrating that there was no sequential progression of cells towards hematopoiesis but rather this cocktail of factors directly reprogrammed non hematopoietic cells to blood cells. Also, investigators noted that with a 24 hour pulse of Doxycycline and a chase of 4 days some cells reverted back to mesodermal and endodermal fate which was shown by expression of brachyury and FoxA2 to previous level. However the ectodermal fate was not recovered as

shown by the absence of Pax6, Sox2 and Otx2 [54]. From the study conducted it was not clear if this combination of transcription factors is sufficient to reprogram fully differentiated somatic cells e.g. fibroblasts. Direct conversion of somatic cells to blood cells may require removal of epigenetic marks and additional time and factors. Even though obtaining desired cell types from pluripotent cells has many implications it may be more advantageous to directly convert one cell type into another to avoid making unwanted cells, intermediate cells or teratomas. Using a simpler, scalable system like monolayer differentiation for production of desired cell types has lot of desirability in therapeutic applications. This system is less variable and can be precisely controlled.

Significance of lineage specific differentiation to blood cells:

Hematopoietic stem cells are important for treatment for disease like leukemia, cancer and genetic disorders of blood. Lineage specific reprogramming to blood has the potential to solve the issue of auto immune reaction during allogeneic transplantations. Direct reprogramming prevents the intermediate un-matured or unwanted cells which carry the risk of tumor formation. They can be used in studying hematopoietic development and in cell and gene therapies.

MATERIALS AND METHODS

Cell Lines:

Inducible SCL-LMO2-GATA2, iSCL, iLMO2 and iGATA2 mouse ES cell lines were created using Ainv15 ES cells lines [18, 66] and p2lox targeting plasmid by Dr. Rita Perlingeiro.

Inducible System:

The Tet Inducible system can be used to understand the effect of desired genes by expressing it at specific time during development. Most of the inducible system use *Escherichia coli* tetracycline resistance operon. In this system, tetracycline repressor transactivator (rtTA) (VP16-mutated TetR fusion) binds to its operon (TetO) driving the expression of target gene through the transcriptional activator by administration of doxycycline. The system used here is a dox-on system that has the advantage of controlling the timing or amount of expression of the target gene depending on the usage of doxycycline. This avoids the toxic effect that could result in gene suppression due to continuous expression of gene or doxycycline. The system uses lox-in strategy that enables easy and efficient integration of any gene of interest. Also, by inverting the orientation of inducible locus it prevents gene silencing and because of the known targeted sites it is easy to retarget if the system requires any modification. [55].

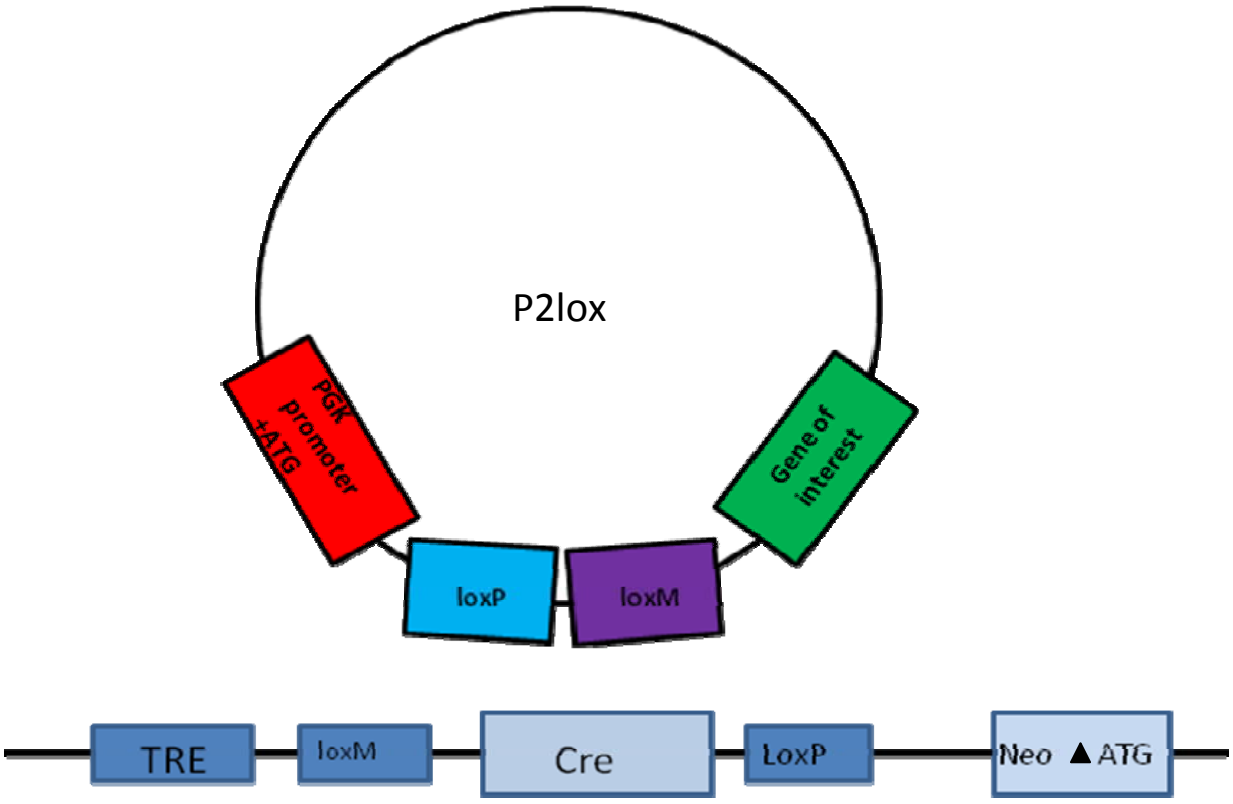


Figure 1. 1: The inducible construct: Illustration of p2lox plasmid and HPRT locus of A2lox.cre cells where targeting sequence has been inserted. Induction of Dox expresses cre before targeting and cre cDNA is removed after lox recombination [5].

Culturing ES cell lines:

ES cell lines are maintained undifferentiated on mouse embryonic fibroblast (MEFs) in ES medium containing Dulbecco's modified eagle's medium (DME) supplemented with 15% fetal bovine serum (FBS), 0.1mM nonessential amino acids (GIBCO), 2mM glutamax (Invitrogen), penicillin/streptomycin (Gibco), 0.1mM β-mercaptoethanol, and 1,000U/mL LIF (Millipore), at 37°C in 5% CO_2. The cells are fed everyday and passaged every two days or when confluent.

Differentiating ES cells in monolayer:

ES cells on MEFs were trypsinized using 0.25% trypsin and added to a gelatin coated well to remove MEFs for 20 – 30 minutes. 15,000 – 20,000 cells were plated on to 0.1% gelatin coated corning plates in ES medium. The medium was changed to EB differentiation medium supplemented with Iscove's modified Dulbecco's medium (IMDM) supplemented with 15% FBS, 200μg/mL iron-saturated transferrin (Sigma), 4.5mM monothiolglycerol (MTG) (Sigma), 50μg/mL ascorbic acid (Sigma), penicillin/streptomycin (P/S) (Gibco), and 2 mM glutamax at 37°C in 5% CO_2, 5% O_2 next day.

For serum free culture, plates were coated with matrigel (BD Biosciences) suspended in DMEM for 1 hour. Around 15,000 – 20,000 cells were counted and plated in ES medium. The medium was changed to growth factor free MtesR1 next day (Stem Cell Technologies) containing MtesR1 basal media and 5X supplement. Additional supplements like P/S, glutamax (2mM), transferring (200μg/mL), ascorbic acid (50μg/mL) and MTG (4.5mM) were added to the media. The cells were fed everyday.

Hematopoietic Colony forming assay:

Colony forming assay is a functional assay which helps in quantification of hematopoietic progenitors. For this assay cells are plated in low density in a semi solid medium like methylcellulose, this medium allows limited cell movements and individual cell clones can be identified as single colony [56]. For the development of hematopoietic colonies the medium requires colony stimulating factors like, stem cell factor (SCF), thrombopoietin (TPO), erythropoietin (EPO), interleukin6, interleukin3. The colonies are counted after 6 days in culture. The colonies obtained are called the colony forming units as they are from a single cell. Cytospins from individual colony can be used to identify cells of different hematopoietic lineages.

For the colony assay following protocol was used, cells were disaggregated into single cells and 50,000 cells were counted and re-suspended in 1.35mL methylcellulose medium supplemented with 15% FBS, 1% bovine serum albumin, 10mg/mL recombinant human (rh) insulin, 200mg/mL human iron-saturated transferrin, 10mM b-mercaptoethanol, 2mM L-glutamine, 50ng/mL recombinant murine stem cell factor, 10ng/mL recombinant murine interleukin-3, 10ng/mL rh interleukin-6, 3ng/mol rh erythropoietin (Stemcell Technologies, MCM3434). 150µL of culture medium was added to adjust the total volume to 1.5mL and plated on 35mm non-adherent petri dishes and cultured in 5% CO2, 5% O2 at 37C. Primitive elytroid colonies were counted 6 days later and other colonies after 10 days.

Cytospin Preparation:

Disaggregated cells were counted and 50,000 cells were suspended in 50µL of culture media and centrifuged on slide at 1000rpm for 5 minutes. The slides were then dried and stained with HEMA 3 kit (Fischer Scientific). The slides were placed in fixative for 10 seconds then transferred to solution I containing eosin for 10 seconds and solution II containing methyl blue for 5 seconds. The slides were washed with water, dried and mounted using wet mount. Hematopoietic cells were observed under the microscope.

Flow Cytometry:

Flow cytometry is a technology that measures and analyzes different physical characteristics of single cells when they flow through a beam of light as a fluid stream [57]. The flow cytometer is made of fluidics, optics and electronics which determine the properties of a cell or particle based on the incident laser light scatter and fluorescence emission. The light scatters based on cell size, granularity and fluorescence intensity. The light scatter is measured either as forward scattered light (FSC) which is proportional to cell surface area/size or as Side scattered light (SSC) which is proportional to cell granularity or internal complexity. A coupled measurement of FSC and SSC along with the staining pattern of cells using different fluorochromes can help differentiate a single cell type in a mixed cell population. Fluorochrome-conjugated antibodies targets the epitope of interest and helps to measure the biological and biochemical properties easily by flow cytometer.

Fluorescent probes are used to quantify or identify different populations of cells or cell surface receptors, for cell sorting, measuring enzyme activity and apoptosis. When using two or more fluorochromes in a single experiment there are chances of spectral overlaps, therefore a process called fluorescence compensation is applied during analysis. The two fluorochromes will display their emission spectra in two channels FL1 and FL2. To calculate the compensation first % of emission spectra of dye A in channel FL2 is detected then % of spillover of dye B is detected in channel FL1. Then using the mathematical formula actual reading for each fluorochrome is calculated.

For fluorochrom A = (total fluorescence measured in FL1) minus (5% fluorochrom B's flouresence)

Similarly, for fluorochrom B = (total fluorescence measured in FL2) minus

(17% fluorochrom B's flouresence) [57]

Gating option is used during data analysis which is a graphical boundary to define the characteristics of a population which will be included for further analysis. This helps to visualize the cells of interest while eliminating dead cells or other debris. For this study data is mostly represented as a two parameter dot plot with a quadrant marker. The percentage of each population in the quadrant is calculated by dividing the percentage of population in one quadrant by the percentage of total gated events. First the gates are applied based on the side scatter and forward scatter were most of the dead and cell clumps are eliminated. Dead cells have lower forward scatter and higher side scatter.

The population selected is called P1. Next gate is setup based on FSC-width and FSC-height; this basically eliminates all the doublet cells and leaves a population

of single cells. As the doublets have more width compared to the single cells and signal emitted by them is low compared to the single cells, which is measured by height. The same is repeated with SSC-width and SSC-height and the population selected is P2 and P3. Next a dot plot consisting of PI (propidium iodide) and PE is setup. This is basically to eliminate dead cells which take up PI stain and are eliminated leaving a population of P4. We can use one or two fluorochromes conjugated to antibodies and obtain desired cell population from P4, which is pure single cell population.

FACS sorting helps us to capture desired population of cells for further analysis. The collected cells can be analyzed biochemically and functionally. To sort the desired population of cells a gate is placed on the cell population in the desired quadrant. The sort gates identify the cells of interest to be sorted out of the stream.

Following protocol was used for preparing cells for FACS analysis and sorting.

The cells were trypsinised using 0.25% trypsin and counted. The disaggregated cells were suspended in staining medium containing PBS and 3% FBS. For flow cytometry analysis, the following antibodies were used: c-Kit-allophycocyanin, CD41-phycoerythrin (PE) from BD Bioscience (0.5µL for $1x10^6$ cells). The cells were incubated in dark on ice for 30 minutes. The cells were then washed with the staining medium and re-suspended in staining medium containing PI and analyzed on a FACS Aria.

RNA Extraction:

The cells were trypsinied and centrifuged. To the pellet 500µL of Trizol (invitrogen) was added. The RNA was extracted using the Invitrogen RNA pure kit. To each of the cell sample 250µl of chloroform was added and centrifuged at 12,000g for 15 minutes. The clear supernatant was transferred to fresh 1ml centrifuge tube. Equal amount of 70% ethanol was added and transferred to a spin cartridge and centrifuged at 12,000g for 15 seconds. 700µl of wash buffer I was added and centrifuged. The flow through was discarded along with the collection tube. 500µl of wash buffer II was added to spin cartridge and centrifuged at 12,000g for 15 seconds and the flow through was discarded. This step was repeated. The spin cartridge was centrifuged for 12,000g for 1 – 2 minutes to dry the membrane with bound RNA. The collection tube was discarded and the spin cartridge was inserted into a fresh recovery tube. 30µl of Rnase free water was added to the center of spin cartridge and incubated for 1 minute and centrifuged at 12,000g for 2 minutes at room temperature to elute the RNA into the recovery tube. The quantity and quality of RNA from each sample was determined using Nanodrop Spectrophotometer ND700. RNA was stored at -80°C until further use.

RNA Sequencing and Analysis:

RNA-Seq is a method of profiling the complete set of transcripts in a cell and quantifying them at a particular developmental stage or physical condition. RNA-Seq is important for interpreting functional elements of genome. This technology reveals the molecular constituents of cells and tissues for understanding development. In this method of transcriptomics, total RNA is converted to cDNA

libraries. Adapters are attached either to one end (single-end sequencing) or both ends (pair-end sequencing). A high through put technology is then used to sequence each molecule [67].

The RNA was prepared using the Invitrogen RNA pure kit from different samples. The quantity and quality of RNA was measured using Nanodrop ND 700 instrument. RNA-sequencing was carried out by the Biomedical Genomic Center at the University of Minnesota.

RESULTS

Blood is formed in the posterior epiblast by induction of visceral endoderm [58]. EB formation is one of the ways of differentiating cells towards the hematopoietic lineage. EB formation mimics embryo development, induction of mesoderm, interaction of epiblast cells and visceral endoderm cells and can therefore form blood when ES cells are differentiated as EB's. Serum plays an important role in mesoderm induction. Therefore media without serum and added growth factors will give much smaller amounts of mesoderm and no blood.

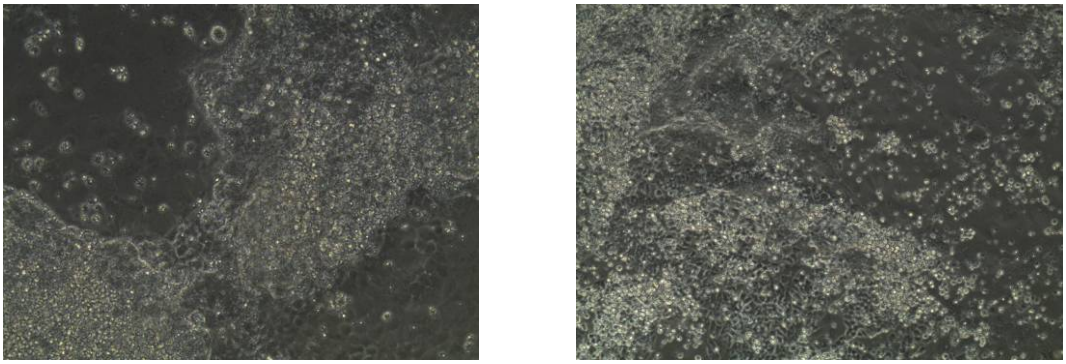
Embryonic development drives the cell towards a particular linage where certain genes are activated and some are silenced. Many factors and signals also play an important role in driving the expression. Indian hedge hog (Ihh) [59] a signaling molecule has been shown to induce hematopoietic factors SCL, LMO2, GATA2 by up regulating BMP4 expression in Xenopus embryos. Studies have shown that SCL, LMO2 and GATA2 are involved in early hematopoietic development and expression of these factors is sufficient for blood formation in animal explants. In this part of my study I co-expressed SCL, LMO2 and GATA2 factors and observed that these factors induced the blood lineage rapidly in the majority of cells even though the induction was for a short duration. This data also suggests that changes induced by thesefactors are stable

1. A 24 hour pulse of SCL-LMO2-GATA2 induces hematopoiesis

I used inducible SCl/LMO2/GATA2 ES cells and induced them with 500ng/mL of Doxycycline on day 3 of monolayer differentiation for 24 hours and then analyzed them on day 6 after 2 days of chase. Expression of inducible genes for a short period was sufficient for production of a significant amount of hematopoiesis compared to the uninduced sample. A colony assay was also performed as a functional assay at day 6.

On day 6 of analysis there was an obvious morphological difference in response to the transcription factor cocktail expression. In the samples induced for just 24 hours the cells were rounded and lifted off the plate, suggesting a hematopoietic fate, compared to the control uninduced samples [Figure 2.1]. CD41 positive cells were seen in the Doxycycline induced sample as indicated by FACS analysis. This observation suggests that short pulse of expression of hematopoietic genes followed by a chase is sufficient to drive the stem cells towards blood formation [Figures 2.2].

A colony assay was performed using methylcellulose and colonies were scored on day 6. The number of erythroid colonies was greater compared to the number of mixed colonies [Figure 2.3]. This correlates with more CD41+ cells than c-Kit+/CD41+ progenitor cells observed in FACS analysis. This was followed by cytospin preparation to characterize cells from individual colonies. The colony assay showed that there were more burst forming unit-erythoid and colony forming unit-erythoid colonies [Figure 2.4].



(a) Uninduced day 6 (b) Dox induced day 6

Figure 2. 1: 24 hour pulse of SCL-LMO2-GATA2

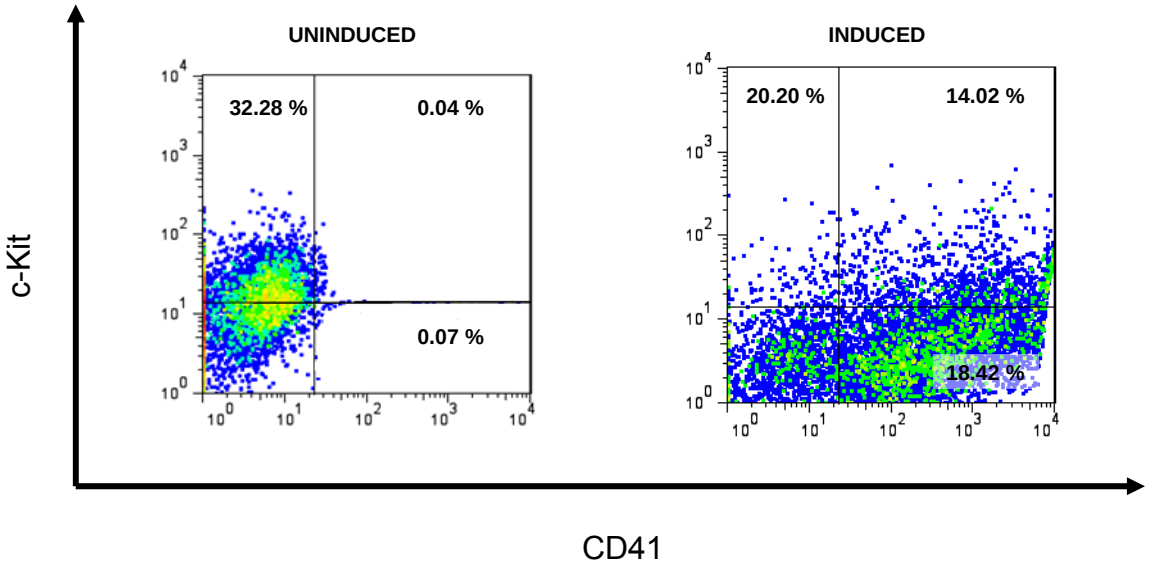
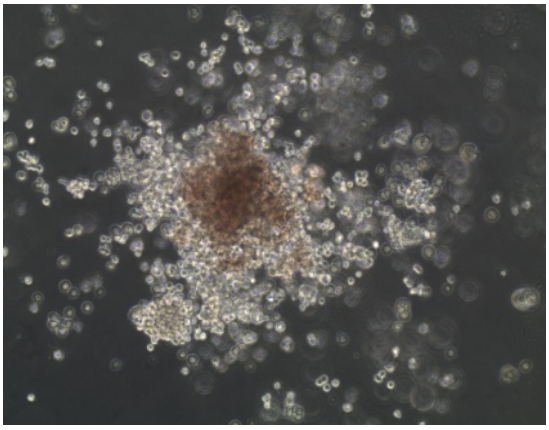
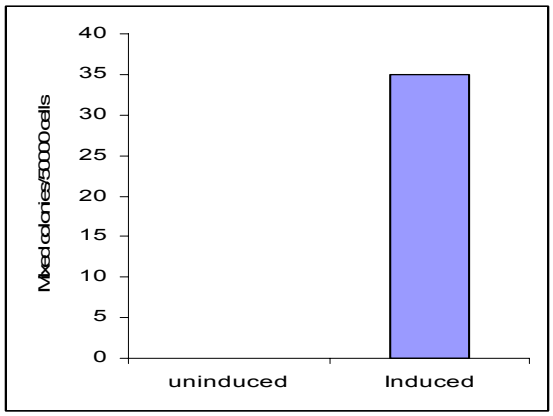


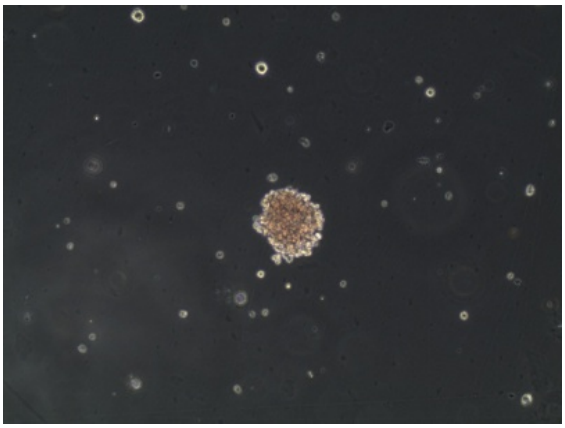
Figure 2. 2 : Day 3 induction of SCL-LMO2-GATA2 for 24 hr - FACS analysis on day 6



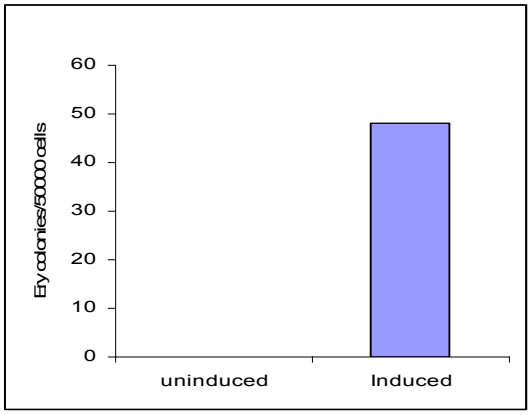
(a) mixed colony



(b) mixed CFC units

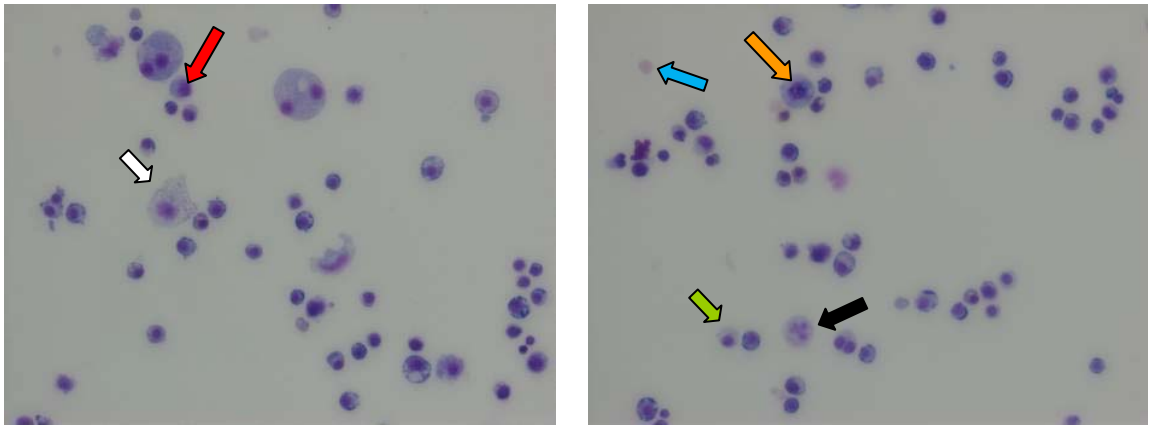


(c) erythroid colony

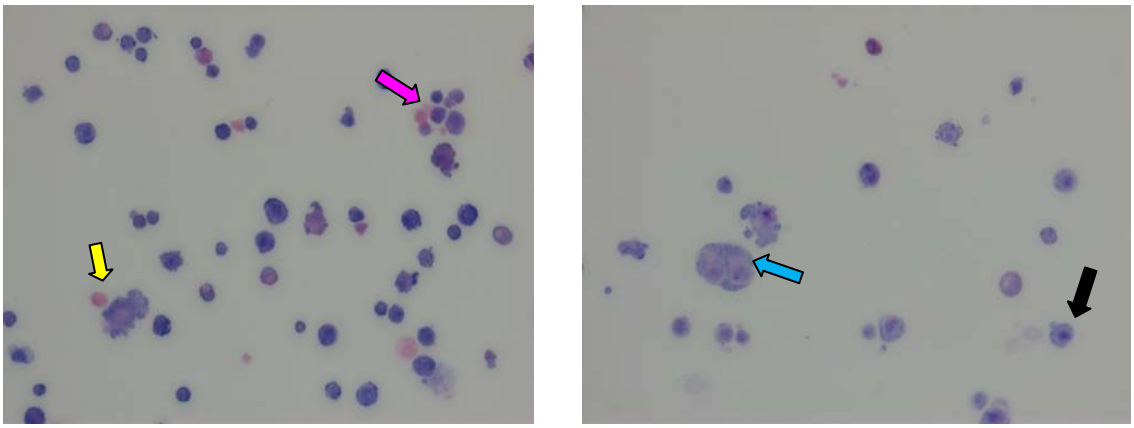


(d) erythroid CFC unit

Figure 2. 3: Colony assay from induced and uninduced samples: Erythroid and mixed colonies were formed in the Dox induced samples on day 6.



(a) cytospins from mixed colony



(b) cytospins from erythroid colony

Figure 2. 4: Cytospins from mixed and erythriod colonies

(a) macrophage (white), lymphocyte (red), monocyte (orange), mature erythrocyte (blue), primitive erythrocyte (green), neutrophil (black) (b) mature erythrocyte (yellow), primitive erythrocyte (pink), megakaryocyte (blue), monocyte (black)

2. Colony Forming Activity of c-Kit+ and CD41+ fractions

Hematopoiesis is a dynamic process of blood cell production and maturation in bone marrow [60]. The cells mature gradually from one stage to the next. CFC assay is an assay for quantifying the committed hematopoietic progenitor cells. The assay is defined by the ability of the progenitor cells to proliferate and differentiate into colonies in a semi-solid media due to cytokine stimulation. The colonies formed are counted between 6 -12 days and characterized according to their morphology.

Hematopoetic cells have different phenotype than the ES cells. Fully matured hematopoietic cells are round and lift off the culture plate. FACS analysis shows that such cells express CD41, a strong hematopoietic marker [57]. CD41 expression also demonstrates early embryonic stage of hematopoiesis.

To investigate in what cellular fraction the hematopoietic colony forming cells arose I induced iSCL-LMO2-GATA2 cells on day 2 of differentiation in serum free media with 500ng/mL of Doxycycline. On day 5 of culture I observed that most of the cells in the Doxycycline induced wells were rounded and lifted off the plate. I did not see this type of morphological difference in the uninduced samples. The floating and attached cell fractions were collected separately on day 5 of culture. I used cKit and CD41 antibodies to sort single positive (CD41), double positive (CD41/c-Kit) and Doxycycline negative control population of cells. I observed that about 90% of floating cells and more than 25% of attached cell were CD41+ [Figure 3.1]. Cytospins and colony assay were performed with the sorted fraction of cells at the end of the experiments.

Cytospins from the floating and attached CD41+ populations had very similar cells types but the attached CD41+ population had some non-hematopoietic cells which were absent in floating population. This indicates that the floating cells are a more pure population of committed hematopoetic cells. The cytospins from the uninduced cells were very different from CD41+ and CD41+/c-Kit+ cell fractions. The cells were much bigger and were non-hematopoietic in nature [Figure 3.2].

For the colony assays, sorted cells were plated in semi solid methycellulose media and plated on 35mm petri dishes. The colonies were scored on day 7 using a scoring dish and cytospins were performed with selected colonies to observe different cell types in that colony. This assay indicated that floating c-Kit+/CD41+ fraction had highest and floating CD41+ cells had the least number of erythroid colonies compared to all the other CD41+ fractions. Attached CD41+ cells yielded higher erythroid colonies than c-Kit+/CD41+ cells. The number of mixed colonies was less than the number of erythroid colonies in all cell fractions [Figure 3.3].

The erythroid and mixed colonies had the following characteristics:

CFU-E (Colony forming unit- erythroid)

The erythroid colony is a small defined colony consisting of atleast 30-50 cells which can be seen from day 6-7 of culture. Individual clusters consist of tiny, irregular shaped cells. Each cluster normally contains 5 - 8 cells, and the size of the cluster is similar to a single macrophage. A large burst forming unit-erythroid (BFU-E) is usually bright red and is differentiable even without the use of a microscope [60] [Figure 3.4].

CFU-GEMM (Colony forming unit-granulocyte, erythrocyte, macrophage, megakaryocyte)

This mixed colony consists of multi-lineage progenitors that give rise to different lineages including erythroid, granulocytes, macrophages, and megakaryocytes. The colony under the microscope can be identified as reddish colored cells (erythroid) mixed with colorless cells (granulocytes, macrophages, and megakaryocytes) in a single colony. Theses colonies are larger compared to other colonies in the culture dish [61] [Figure 3.4].

CFU-GM (Colony forming unit-granulocyte, macrophage), CFU-M and CFU-G

The CFU-GM colony includes colonies of granulocytes and macrophages. Macrophages are colorless, large, round and vacuolated cells. The clonogenic progenitors of granulocytes will give rise to a population that is colorless and smaller than macrophages, whereas the clonogenic progenitors of both granulocyte and macrophage will give rise to a heterogeneous population of macrophages and granulocytes. These colonies consist of colorless round cells (granulocytes) and oval cells (macrophages). The cells can be distinguished from erythrocytes by the size and color. These colonies appear in the culture at day 7 and can be observed up to day 12 [60] [Figure 3.4].

The reason for lower number of mixed colonies in all the cell fractions could be lack of cytokines and growth factors in the defined culture medium that was used [Figure 3.5]. The experimental results show that the serum free media most likely does not maintain hematopoietic progenitor cells and the cells are prone to

differentiate to a committed erythroid lineage. Further experiments were conducted using additional cytokines and growth factors which were supplemented with the culture media to obtain more primitive hematopoietic cells

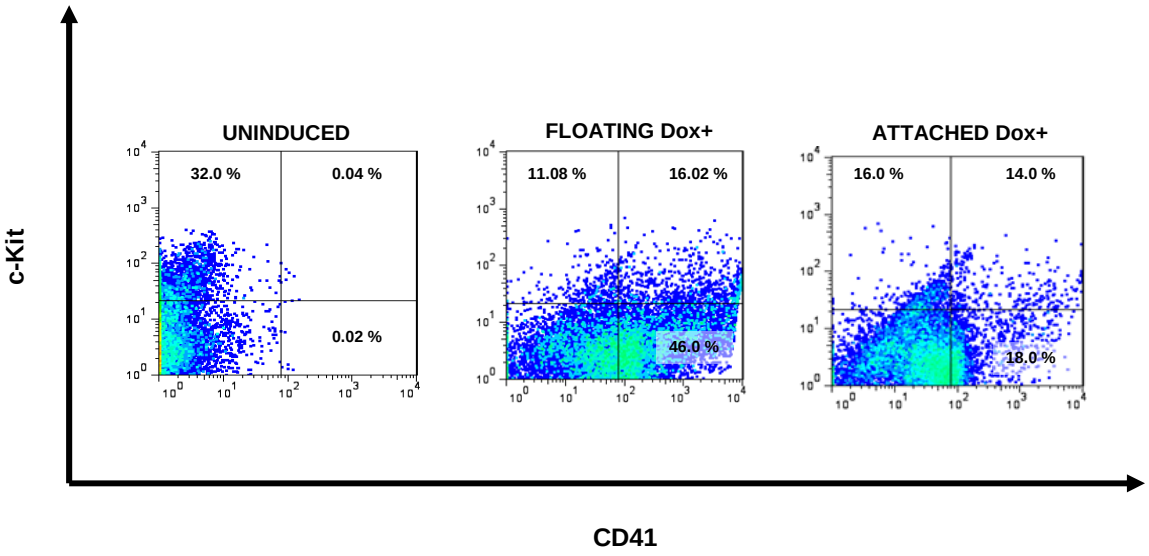
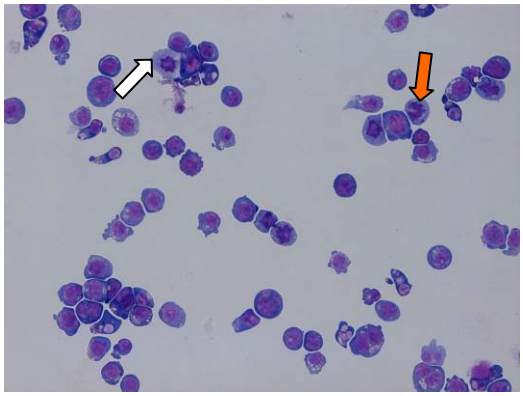
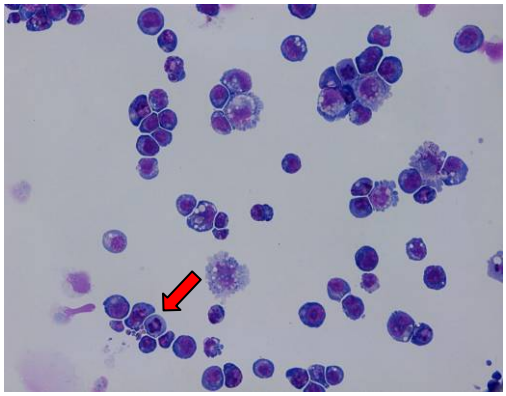


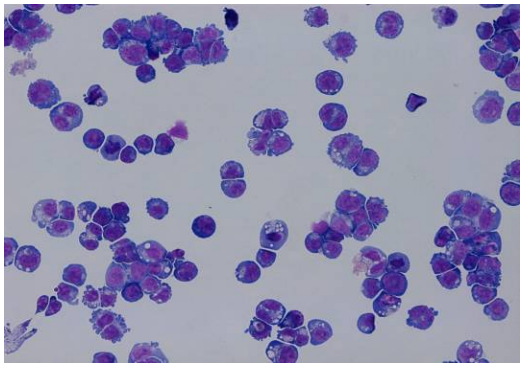
Figure 3. 1: Day 2 induction of SCL-LMO2-GATA2 for 24 hr - FACS analysis on day 5 (floating and attached cells)



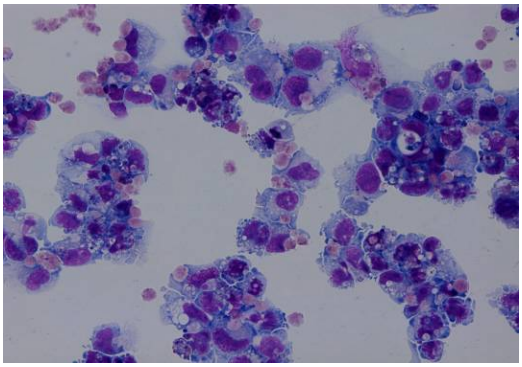
(a) floating CD41+



(b) attached CD41+



(c) attached c-Kit+/CD41+



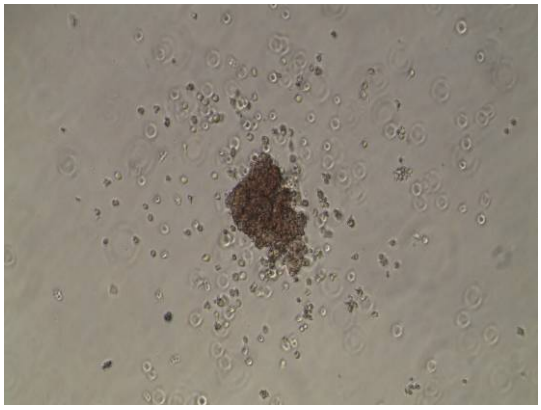
(d) uninduced control

Figure 3. 2: Cytospins from sorted fractions

(a) Monocyte (white), band neutrophil (orange) (b) band neutrophile (red)

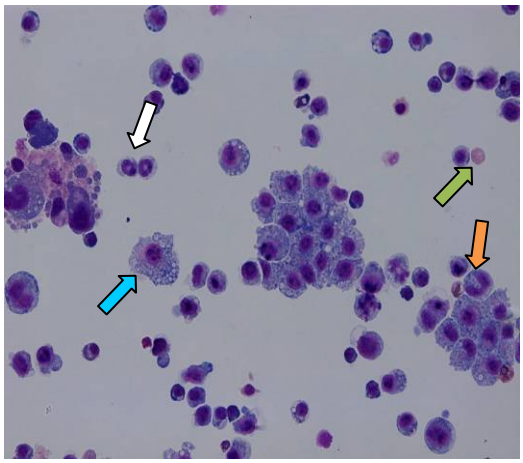
(a) CFU-GM

(b) CFU-GEMM

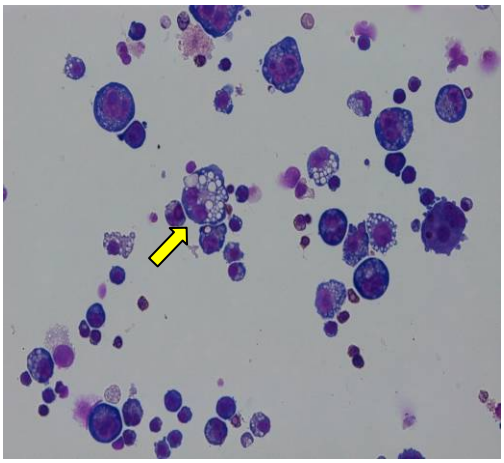


(c) CFU-E

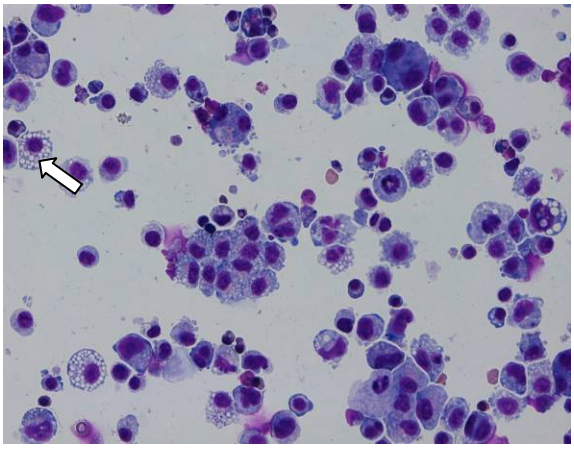
Figure 3. 3: Mixed (a) and (b) and Erythroid (c) colonies in colony forming assay obtained from attached CD41+ (c), attached c-Kit+/CD41+ (b) and floating CD41+ (a)



(a) CFU-GM



(b) CFU-GEMM



(c) CFU-E

Figure 3. 4: Cytospins from mixed and erythroid colonies

(a) Macrophage (blue), mature erythrocyte (green), band neutrophil (orange), monocyte (white) (b) Macrophage (yellow) (c) Macrophage (white)

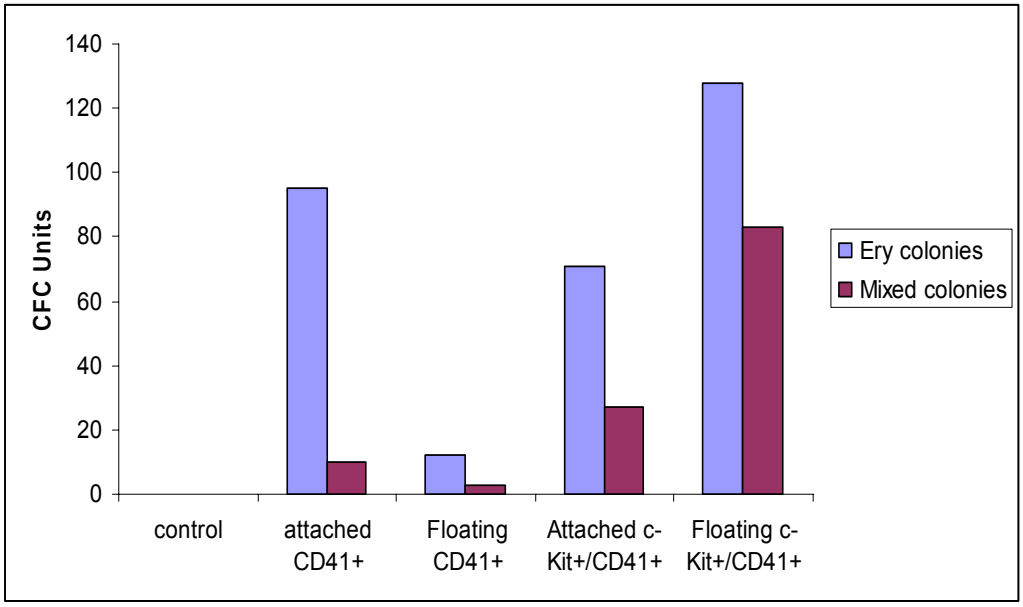


Figure 3. 5: Colony forming units obtained from sorted cell populations (day 6)

3. Use of growth factors to capture early progenitors

The inducible SCL/LMO2/GATA2 cells differentiate towards the hematopoietic lineage very efficiently. Studies conducted by Ismailoglu [5] demonstrated that day 4 inductions for 24 hour followed by chase for one day and day 6 analysis by FACS for c-Kit/CD41 showed high expression of CD41 (about 50%) single positive cells [5]. Hematopoietic cells are most commonly cultured in media containing cytokines and growth factors neither of which were present in the serum free media used in my experiment. The absence of the serum could make the cell differentiate more rapidly once they are committed to the hematopoietic fate. Also, SCL and LMO2 are important for maturation of erythrocytes and megakaryocytes and may be promoting these lineages resulting in forming more erythroid colonies compared to mixed colonies.

To capture the early progenitors a number of growth factors were supplemented with the growth factor free media including:

Stem cell factor (SCF- 50ng/mL): SCF is a stromal cell derived factor synthesized by many cell types including hematopoietic cells. In vitro administration of SCF increases primitive lymohoid and myeloid hematopoietic bone marrow progenitor's cells expressing the early surface marker Sca-1. SCF increases number of megakaryocytes and circulating platelets. SCF along with EPO increases proliferation of erythroid colonies. It also has synergistic role with GM-CSF, IL-6, TPO in multipotent colony formation [61].

Thrombopoeitin (TPO-40 ng/mL): TPO is a growth factor for the HSC. It also functions as a natural stimulator to increase the number and size of

megakaryocytes by increasing the number of the small acetyl-cholinesterase that is early precursors of megakaryocytic lineage [61].

Interlukein 6 (IL6- 6ng/mL): IL6 promotes the proliferation of multipoetent hematopoietic progenitor cells. It stimulates the production of macrophages, mast cells, gial cells, T-cells B-cells and many other cell types. It behaves like TPO and helps in maturation of megakaryocytes in vitro [61].

Interlukine 3(IL3-20ng/mL): IL3 helps in proliferation and differentiation of most types of hematopoietic progenitor cells. IL3 stimulates the colony formation of granulocytes, megakaryocytes, macrophages, erythrocytes from non lineage committed hematopoietic progenitor cells. It also helps in priming of hematopoietic stem cell in vitro and in vivo thus making them respond to later acting factors like EPO, IL-6 and GM-CSF [61].

Erythropoietin (2u/mL): Epo stimulates the differentiation and maturation of erythrocytes. It also helps in regulating proliferation of erythropoeitic progenitor cells. EPO increases the colony numbers of BFU-E, CFU-E. EPO treatment leads to expansion of thrombopoietic progenitor cells [61].

Leukemia inhibitory factor (LIF-10ng/mL): LIF supports the terminal differentiation of myeloid leukemia cells. It belongs to IL-6 cytokine family.

The serum free growth factor free media was supplemented with six cytokines mentioned above on day 4 of differentiation and induced with 500ug of Doxycycline for 24 hours followed by a chase of 3 days. The floating and attached cell fractions were analyzed and sorted by FACS for c-Kit and CD41

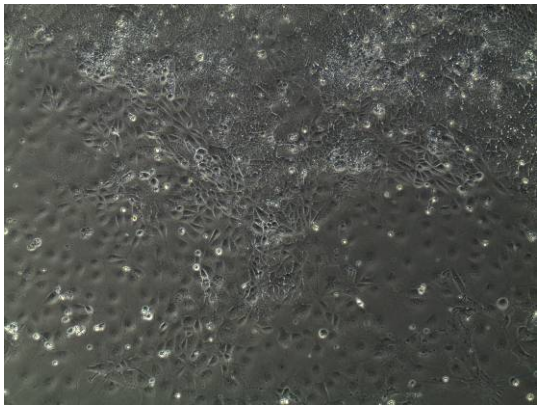
antibodies. Colony assay followed by cytospins were performed on the different sorted populations of cells.

On the day of analysis there was an obvious difference in the cells treated with growth factors [Figure 4.1]. The majority of cells lifted off the plate in cytokine conditioned media. More than 95% of cells were positive for hematopoietic markers [Figure 4.2]. Floating cell fractions had more of CD41+ single positive cells [Figure 4.2]. The colony forming activity of floating cells was very less as only few colonies were obtained from the CD41 single positive and c-Kit/CD41 double positive sorted floating cell population [Figure 4.3].

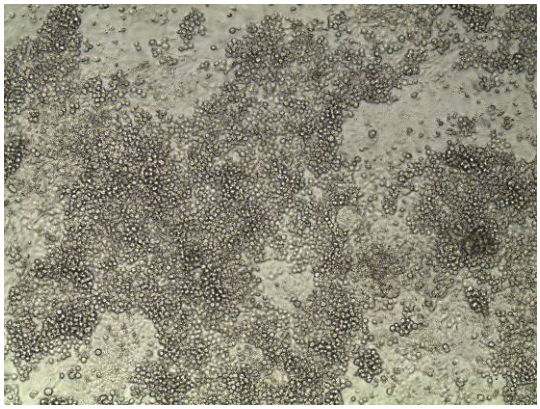
There was not much difference between the growth factor supplemented and non-supplemented attached cell samples as both cell population showed 50% of hematopoiesis [Figure 4.2]. But a difference was observed in the colony forming activity of attached cells. There was a two fold increase in the number of mixed colonies in the cytokine treated [4.3, 4.4] fraction compared to the untreated sample [4.5, 4.6]. Also there was an increase in the overall colony formation in growth factor treated samples [Figure 4.7].

The result obtained may be due to short duration of cytokines supplementation which could have allowed the cells to undergo terminal differentiation once they were committed to hematopoietic fate. Therefore primitive hematopoietic cells were not maintained in the culture, which was further supported by a reduced c-Kit+/CD41+ population compared to CD41+ population. I did not analyze whether the primitive hematopoietic cell population increased if the cytokines were supplemented throughout the culture period.

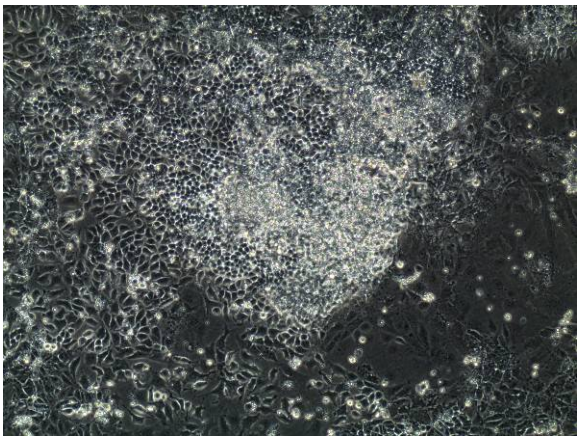
In the colony assay there were more mixed colonies from the cytokine treated c-Kit+/CD41+ sorted populations compared to the untreated samples, implying that cytokines indeed promoted proliferation and differentiation of primitive hematopoietic colonies.



(a) uninduced



(b) induced cytokines (+)



(c) induced cytokines (-)

Figure 4. 1: Day 4 induction of SCL-LMO2-GATA2 for 24 hr – images on day 7

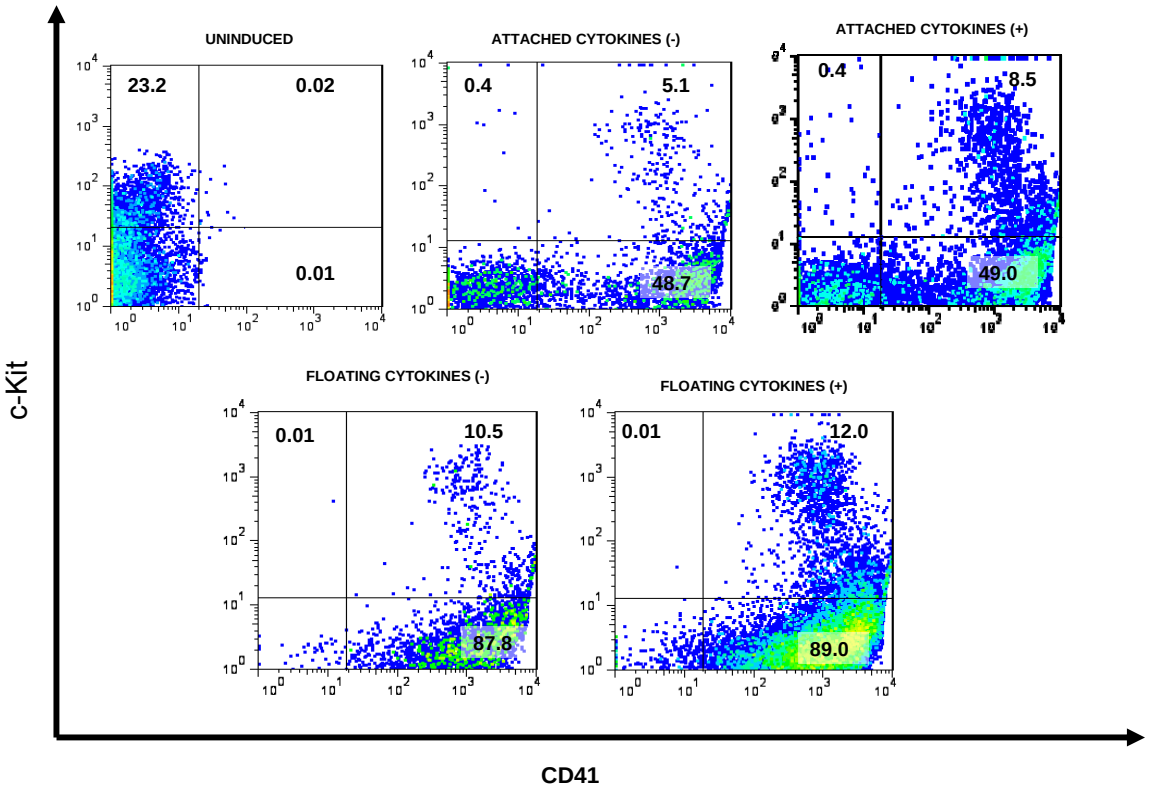
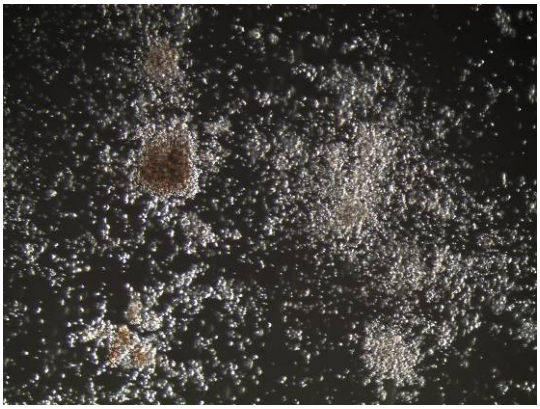


Figure 4. 2: Day 4 induction of SCL-LMO2-GATA2 for 24 hr - FACS analysis on day 7 (floating and attached cells)



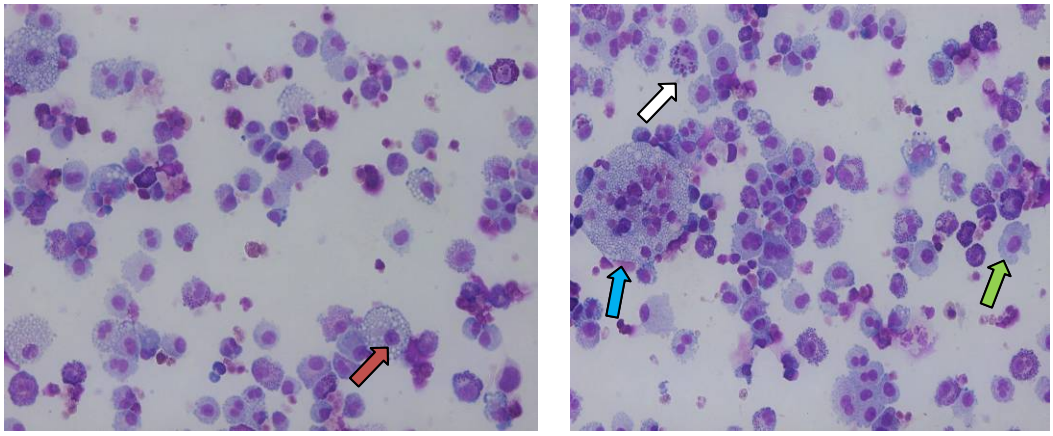
(a) attached CD41+

(b) attached c-Kit/CD41+

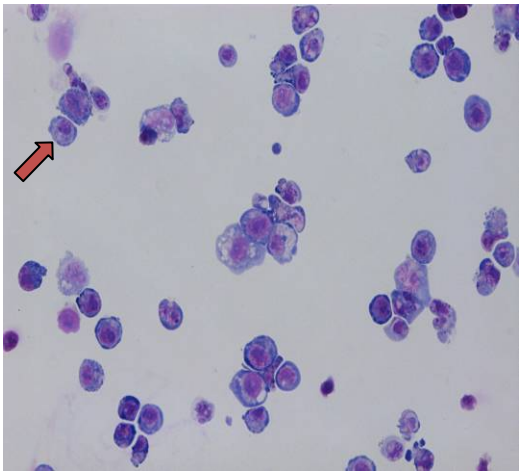
(c) floating c-Kit/CD41+

(d) floating CD41+

Figure 4. 3: Colony assay performed with cytokine (+) Dox (+) attached and floating sorted populations (mixed (b, c) and erythroid (a, d) colonies)



(a) attached CD41+ (b) attached c-Kit+/CD41+

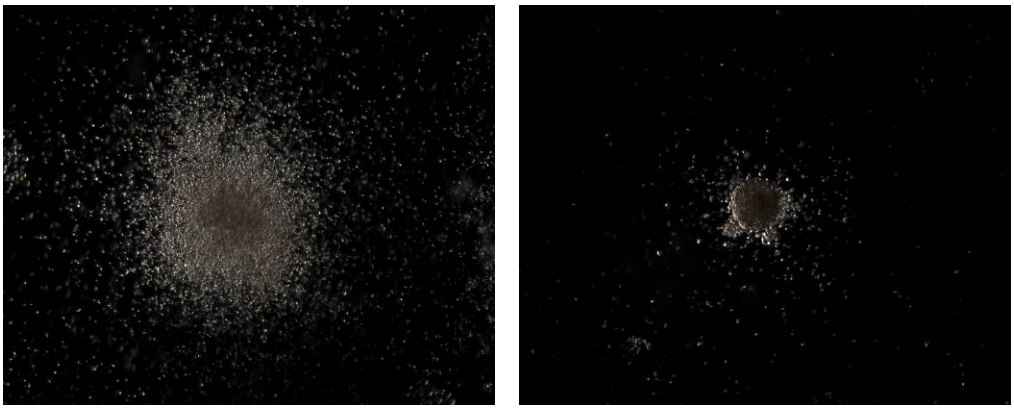


(c) floating CD41+

Figure 4. 4: Cytospins from sorted fractions.

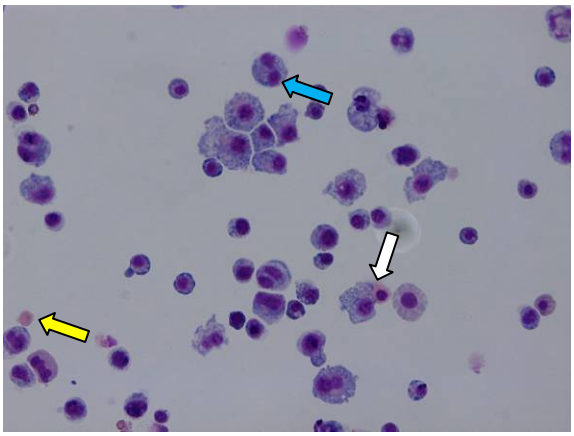
(a) macrophage (red) (b) megakaryocyte (blue), neutrophil (white), primitive erythrocyte (green) (c) erythrocyte (red)

(a) attached CD41+ (b) floating CD41+

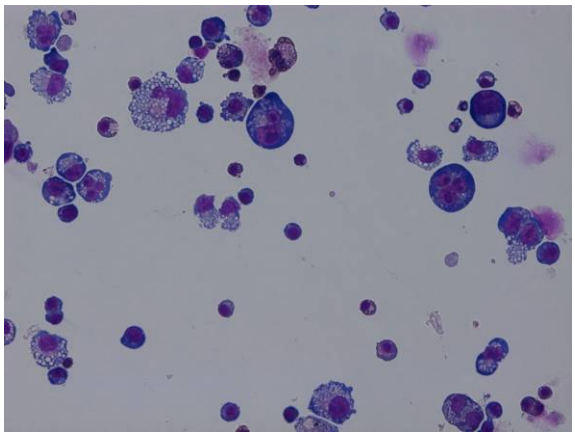


(c) attached c-Kit+/CD41+ (d)floating c-Kit+/CD41+

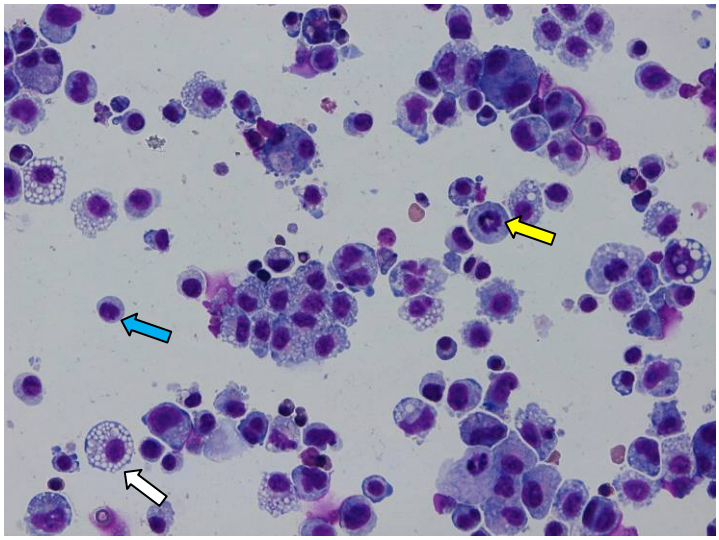
Figure 4. 5: Colony assay performed with cytokine (-) Dox (+) attached and floating sorted day 7 cell populations (images – day 7)



(a) attached CD41+



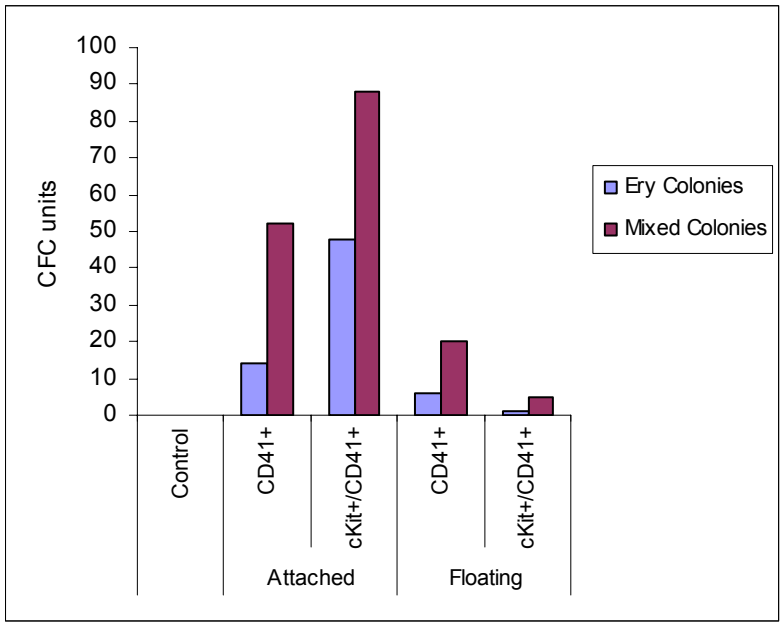
(b)floating CD41+



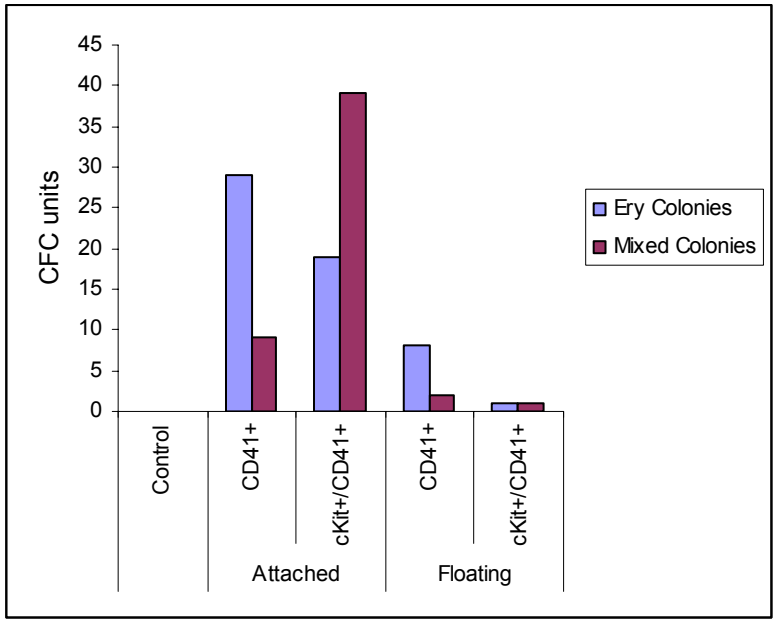
(c) Attached c-Kit+/CD41+

Figure 4. 6: Cytospins from sorted fractions.

(a) matured erythrocyte (yellow), primitive erythrocyte (white) neutrophil (blue) (c) erythrocyte (blue), macrophage (white), neutrophile (yellow)



(a) CFC units from cytokine (+) Dox (+)



(b) CFC units from cytokines (-) Dox (+)

Figure 4. 7: Colony forming cell units obtained from cytokine (+) Dox (+) and cytokines (-) Dox (+) population

4. RNA Sequencing data shows high expression of hematopoietic genes in the SCL, LMO2 and GATA2 induced cells

The RNA sequencing data consisted of RPKM values for around 40,000 genes in the mouse genome for all the samples. RPKM means reads per kilo base of exon model per million mapped reads. RPKM is a measurement of frequency of sequence reads that corrects for the length of the gene. RPKM normalizes the reads according to the length of the gene, i.e. equal number of reads from a short transcripts means higher expression compared to the longer transcript [68].

$$RPKM = \frac{Total\ \ exon\ reads}{Mapped\ reads\ (millions) \times exon\ length}$$

To normalize the doxycycline induced sample to the control uninduced sample, the RPKM value of each gene from different samples (Dox induced) were divided with the uninduced control samples. The data was then transformed using the log transformation. To obtain the top 20 up regulated genes the log values were multiplied to the absolute change value (difference of control and induced sample). The RNA sequencing data consisted of two different sets of experiment. For the first experiment I induced SCL-LMO2-GATA2 cells on day 4 of differentiation and collected the RNA 6 hours and 12 hours after the induction. RNA from uninduced sample was collected as control.

For the second set of experiment, I performed a pulse and chase experiment. I induced the SCL-LMO2-GATA2 cells along with supplementation of cytokines on day 4 for 24 hours. On day 7 of differentiation I sorted CD41+ and c-Kit-/CD41- fractions from attached and floating cells. RNA was collected from all the samples ($1x10^6$ cells). As a control RNA was collected from the uninduced un-

fractioned sample. The RNA sequencing data would be important to understand the differential gene expression between induced and uninduced samples and also to know which kind of cells are being reprogrammed.

The RNA-seq data from the 12 hour induced sample compared to 6 hour showed higher expression of hematopoietic genes including SCL, LMO2, GATA2, Runx1 and globins indicating that short pulse of induction was sufficient for inducing hematopoietic cells (Figure 5.1). The data showed a decrease in the endothelial markers including Hes2, Hes5, ve-cadherins and Notch1, indicating that the SCL complex is promoting the blood lineage but not the endothelial (Figure 5.2). The data also indicated a decrease in ectodermal gene expression (Figure 5.2).

As shown in figure 5.5, floating CD41+ cells had increased expression of hematopoietic genes compared to attached CD41+ suggesting that the floating cells were more committed to hematopoietic lineage compared to the attached cells.

Figure 5.5, compares attached CD41+ cells to the c-Kit-/CD41- population and shows that although c-Kit-/CD41- population expressed most of the hematopoietic genes (low levels compared to CD41+ cells) they failed to make blood cells. The CD41- cells showed increased levels of endodermal markers suggesting that long chase (3 days) following induction reverts the cells to a near by lineage (Figure 5.9). Also, the primitive mesodermal markers like Brachyury and goosecoid (Figure 5.9) were decreased in the CD41+ cells indicating that SCL complex is suppressing endodermal and mesodermal genes and increasing the hematopoietic gene expression. These results prove that at transcriptional

level the triple construct can promote hematopoiesis. The 20 most up-regulated genes included key hematopoietic genes in the 6 hour and 12 hour sample (Figure 5.3) and globins in the sorted fractions indicating a hematopoietic fate (Figure 5.7).

Also, the attached CD41+ and CD41- cells showed decreased levels of ectodermal markers including Pax6, FoxG1, Otx2, Trka, Trkb, Trac, Sox1, Trim33, Ncam1 and Ncam2 (Figure 5.6). This suggests that the SCL complex may be erasing cells of ectodermal lineage efficiently. Further analysis is required to prove that the transcription factor cocktail can be used to reprogram somatic cells of other lineages.

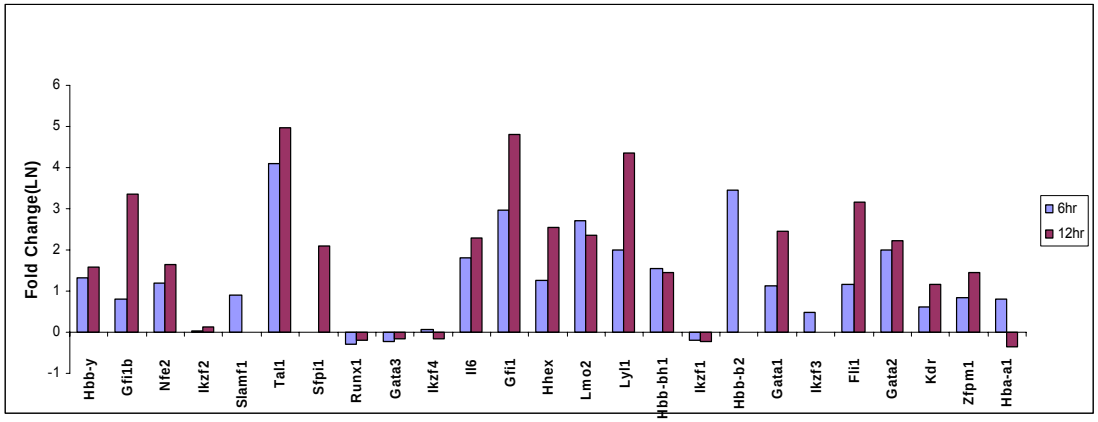
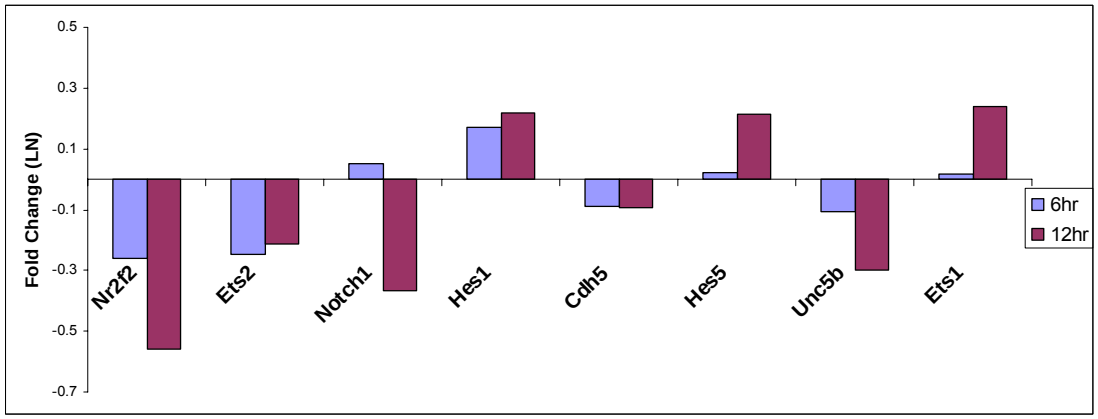
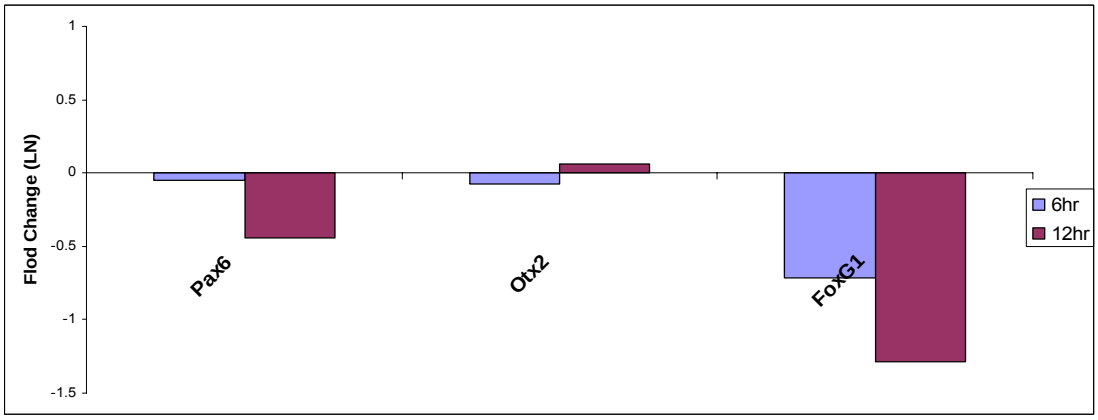


Figure 5. 1: Hematopoietic Gene expression in 6 hr compared to 12 hr samples

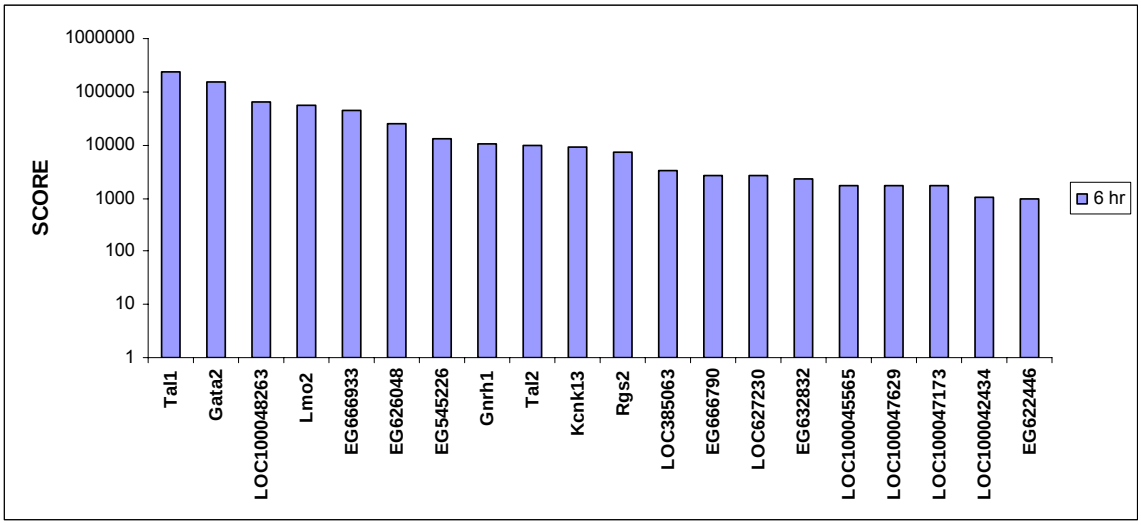


(a)

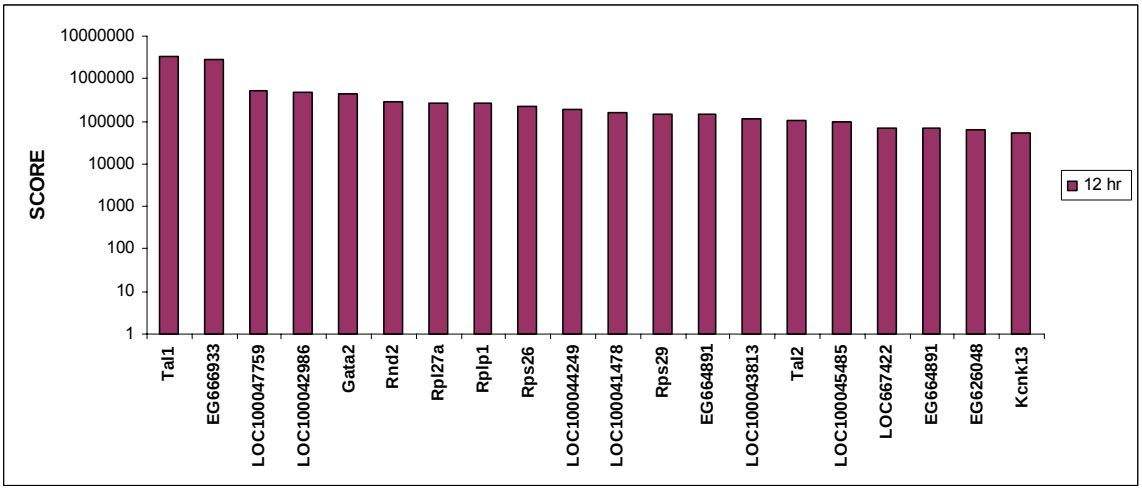


(b)

Figure 5. 2: Endothelial (a) and Ectodermal (b) gene expression in 6 hr and 12 hr induced samples

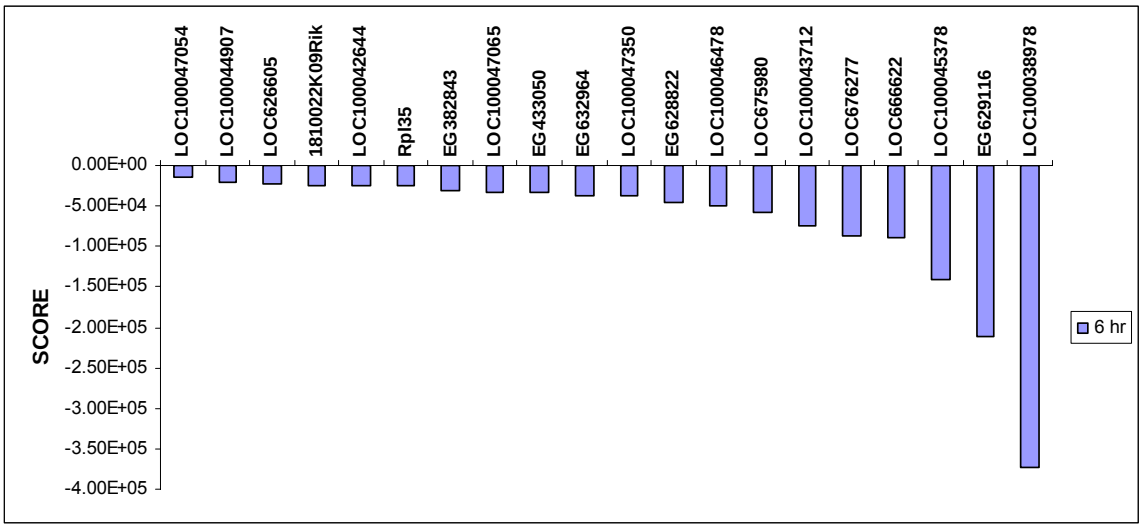


(a)

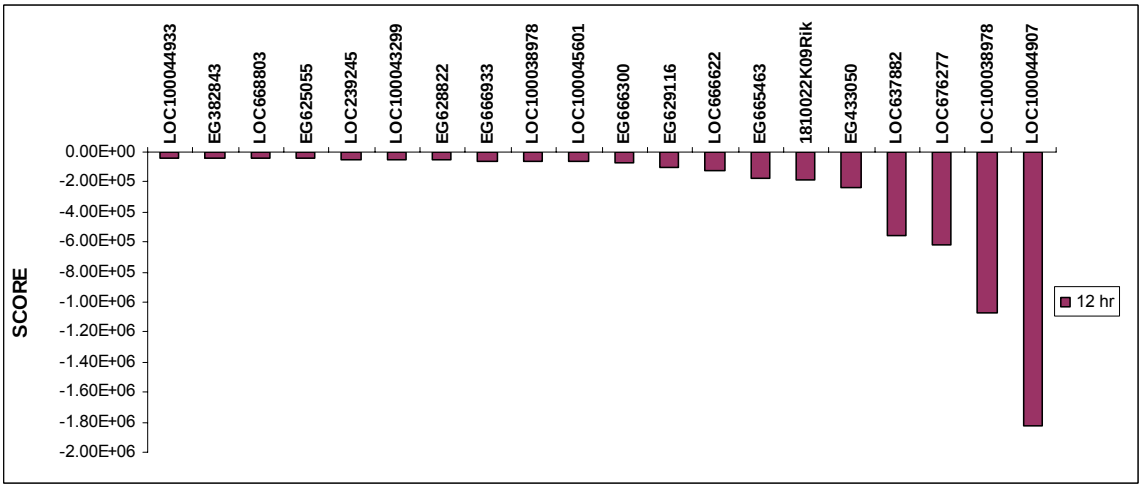


(b)

Figure 5. 3: Top 20 up-regulated genes in the 6 hr (a) and 12 hr (b) induced samples



(a)



(b)

Figure 5. 4: 20 most down regulated genes in 6 hr (a) and 12 hr (b) induced samples

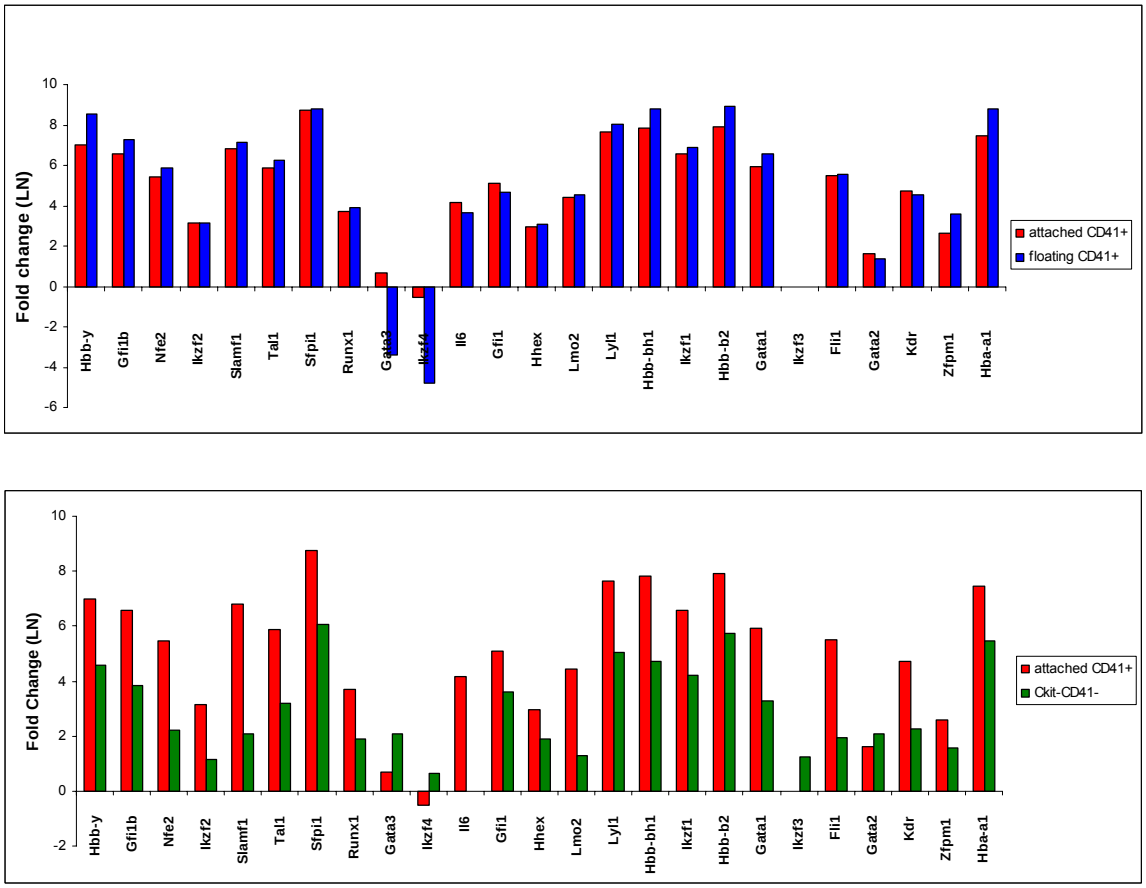
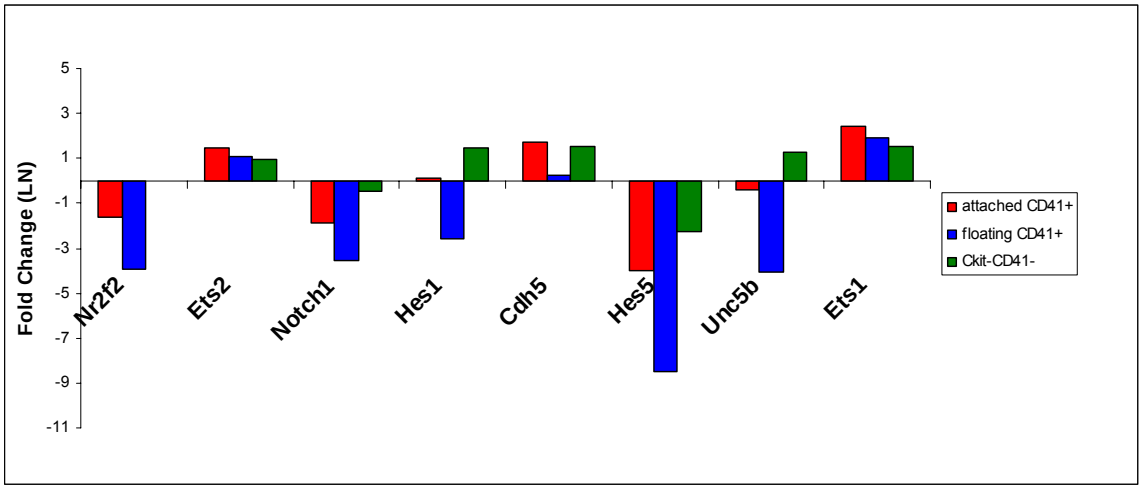
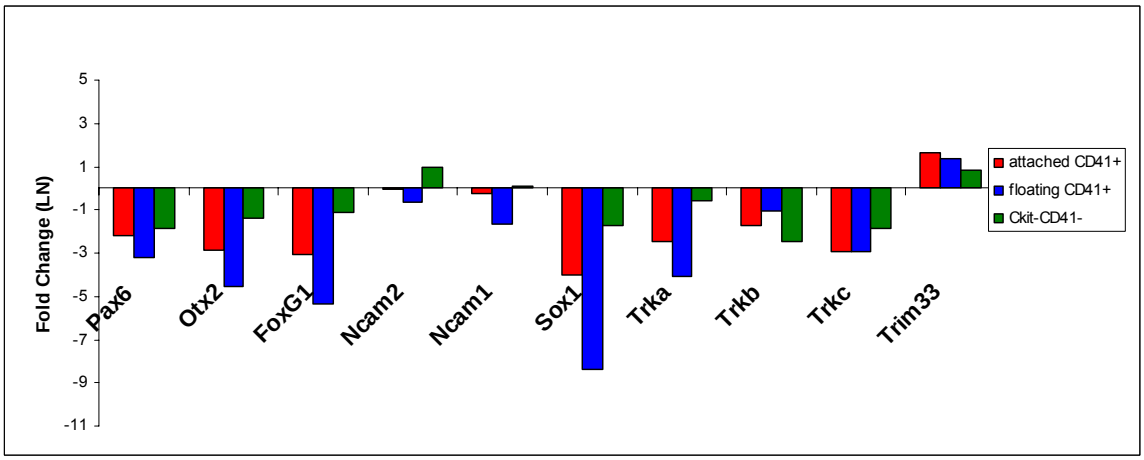


Figure 5. 5: Hematopoietic gene expression in Attached and Floating CD41+ and c-Kit-/CD41- samples

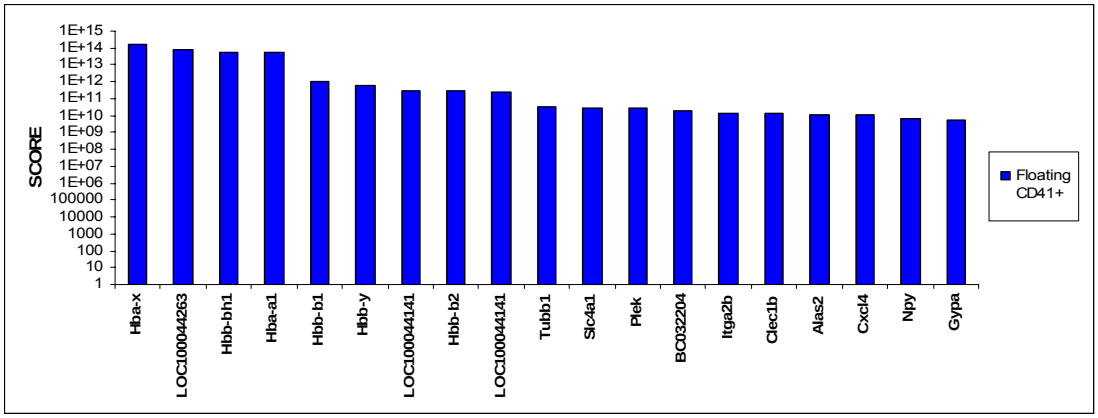


(a)

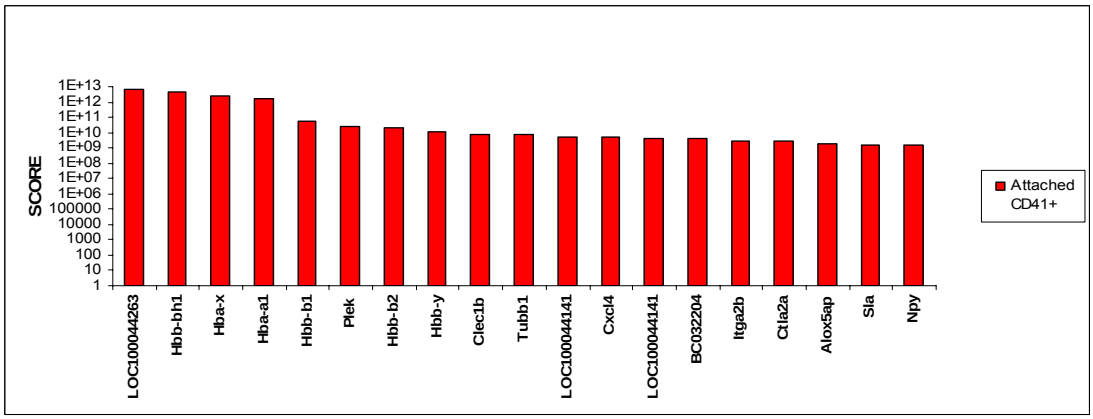


(b)

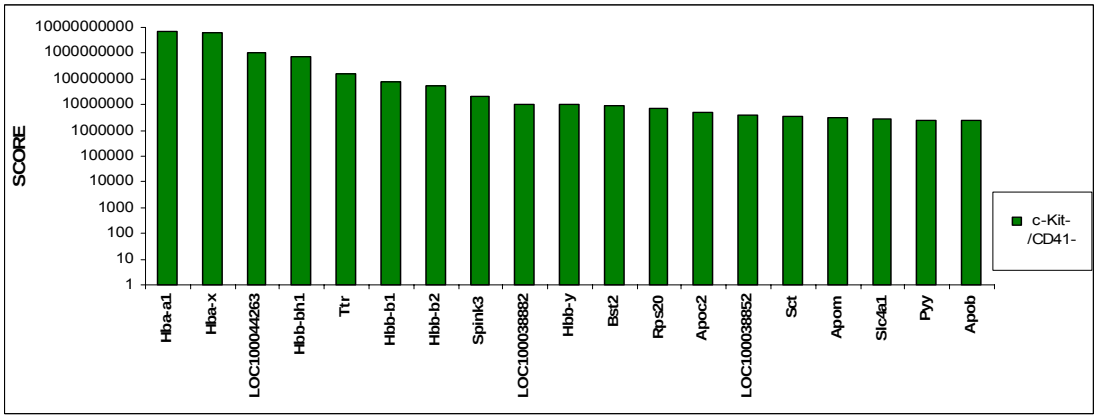
Figure 5. 6: Endothelial (a) and Ectodermal (b) gene expression in Attached and Floating CD41+ and c-Kit-/CD41- samples



(a)

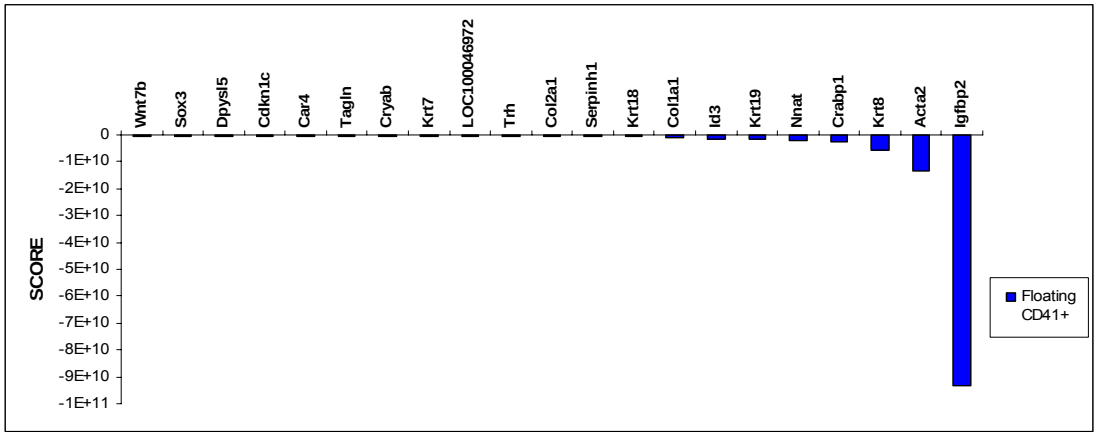


(b)

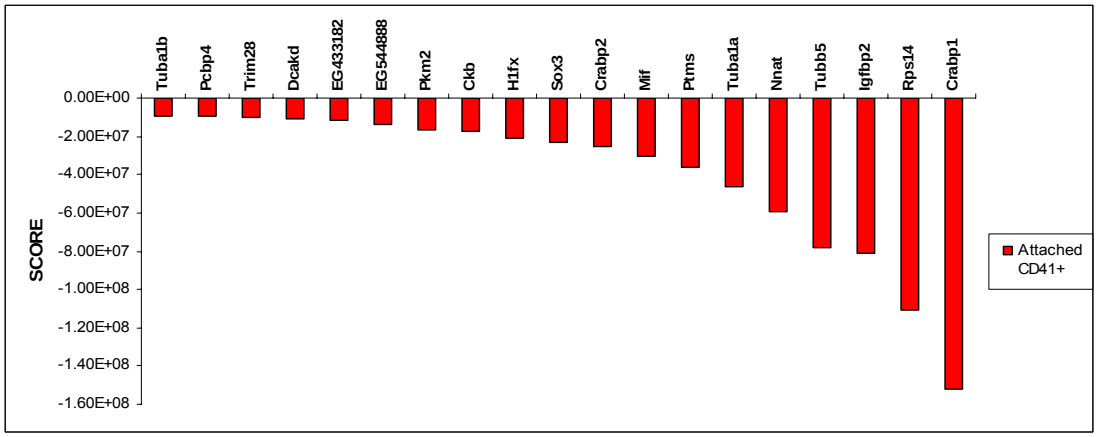


(c)

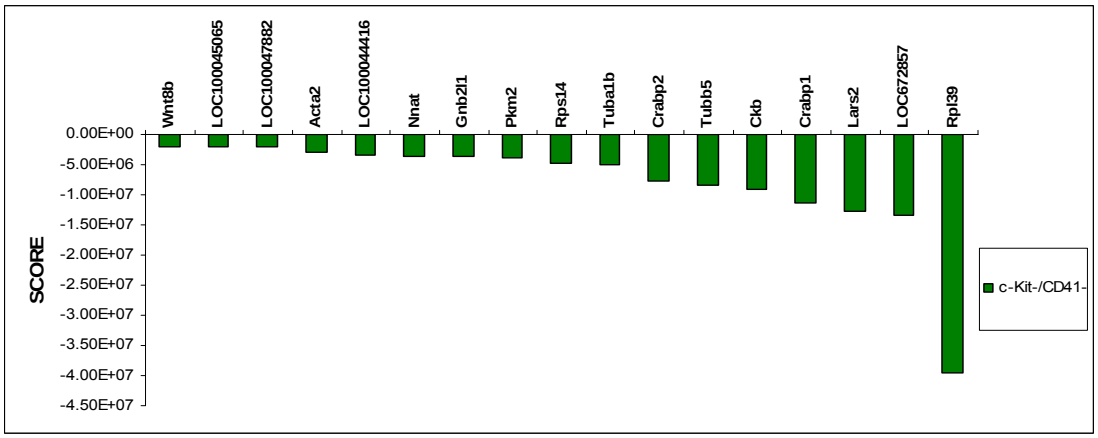
Figure 5. 7: Top 20 up-regulated genes in floating (a) and attached CD41 (b) c-Kit - CD41- (c) samples



(a)

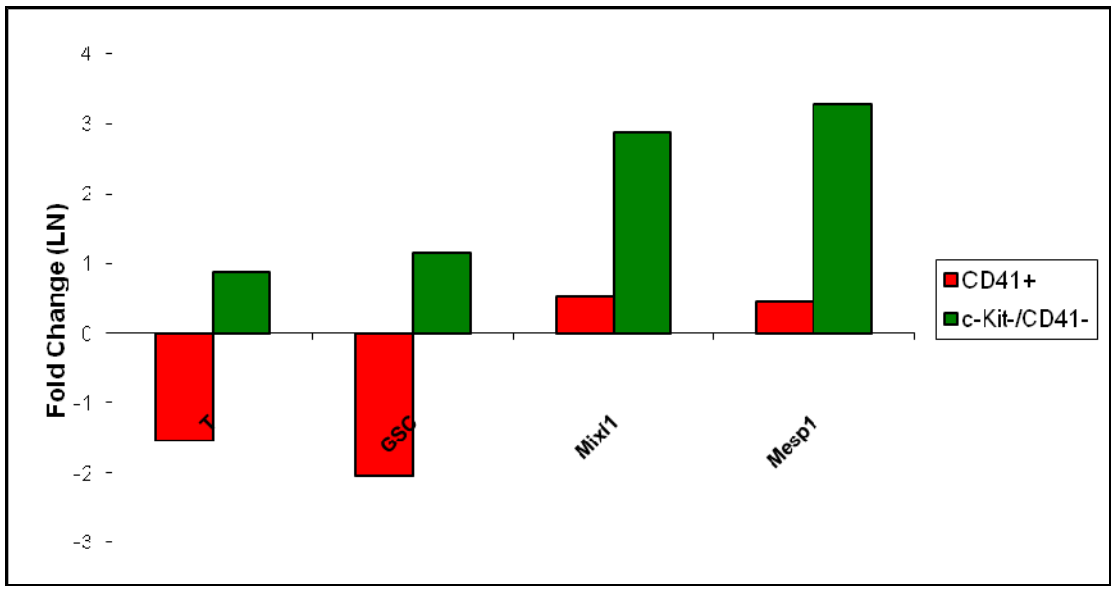


(b)

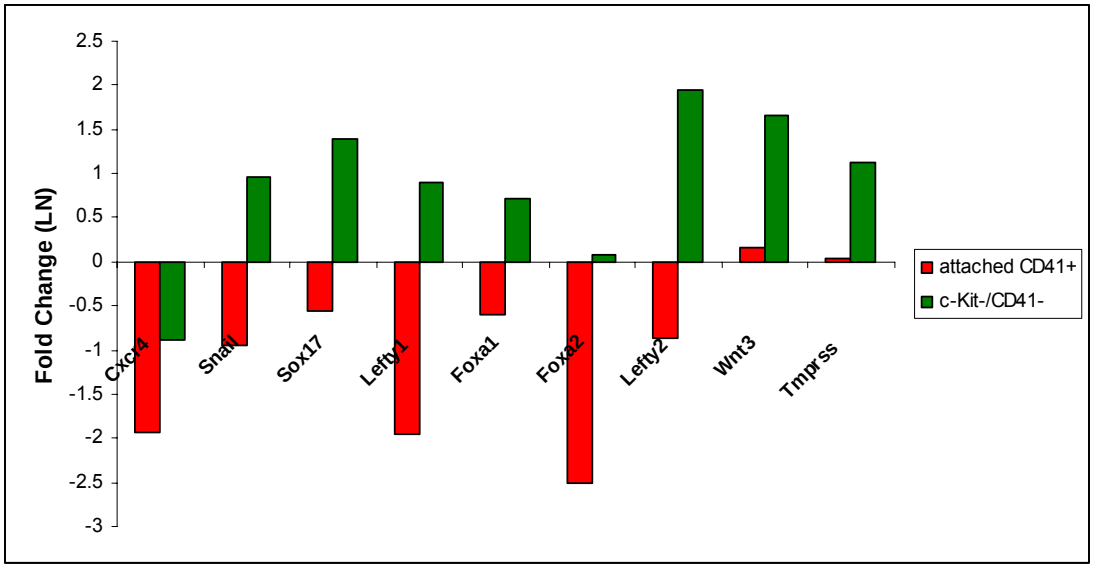


(c)

Figure 5. 8: 20 down-regulated genes in floating (a) attached CD41+ (b) c-Kit-/CD41- (c) samples



(a)



(b)

Figure 5. 9: Mesodermal (a) Endodermal (b) gene expression in CD41+ and cKit-/CD41- samples

DISCUSSION

The study conducted shows the ability of SCL-LMO2-GATA2 to directly induce hematopoietic reprogramming and shed light on the mechanism and nature of the reprogrammed cells. This cocktail of transcription factor reprograms somatic cells derived from pluripotent stem cells to blood cells in a monolayer culture under defined conditioned medium without growth factors.

To address the question if the transcription factor complex was able to stably reprogram ES cells to blood I induced the SCL-LMO2-GATA2 cells for a short pulse of time. A changed cell phenotype along with high expression of CD41+ was observed in the induced sample compared to the control proving a hematopoietic fate. Cytospins from different sorted populations including floating and attached CD41 and c-Kit+/CD41+ fractions showed different types of cells from granulocyocytic, monocytic, megakaryocytic and erythroid lineages demonstrating the ability of the cocktail of factors to induce hematopoietic differentiation. The colony forming assay demonstrated that in addition to differentiated blood cells the SCL complex can generate hematopoietic progenitors. In order to capture the early progenitors in the SCL, LMO2 and GATA2 induced samples, a specific cytokine treatment was applied. This incresed the number of colony forming cells, and the colonies they produced consisted of multipotent hematopoietic and committed progenitors. The results obtained helped in the optimization of the system. Further experiments are required to determine if this system can be used to generate transplantable HSC capable of chimerizing adult recipients.

The RNA sequencing data from the induced samples clearly demonstrated that SCL complex specifically induces hematopoiesis. This proves the reprogramming potential of the cocktail factors even with a short pulse of induction. Surprisingly the SCL complex does not promote endothelial progenitors showing that the way blood is formed in this system is distinct from the way it is formed in the embryo. The data shed light on what the CD41- "non-reprogrammed" cells were. These cells have lower ectodermal gene expression and do express hematopoietic genes, but also expressed higher levels of endodermal genes which was unexpected. Further work is required to answer why the negative cells express all the blood genes, but fail to express the hematopoietic marker CD41. Possible reasons could be either partial reprogramming of these cells or they may be a mix of cell types. Also some cells may take longer to get reprogrammed or they may have turned off the CD41. The expression of ectodermal genes (Pax6, FoxG1, Otx2, Trka, Trkb, Trac, Sox1, Trim33, Ncam1 and Ncam2) Foxa2) was down regulated in the CD41+ cells, suggesting that the SCL cocktail generates blood at the expense of other lineage. Future experiments could involve generating transgenic mouse with an inducible SCL- LMO2-GATA2 cassette. Obtaining blood cells from the tail tip fibroblasts by induction of the SCL complex would help understand reprogramming of somatic cells to blood cells Further analysis of the RNA-seq data could provide more information about the role of other important genes or pathways that are up regulated or down regulated during the reprogramming to hematopoietic lineage. This study also shows that a continuous induction of these hematopoietic-inducing factors is not required and just a pulse of transcription factor complex is sufficient to achieve hematopoietic commitment.

Future experiments are required to asses if the SCL complex can generate HSC. This can be achieved by transplanting the cells obtained from the monolayer culture into adult model animals.Another important step in the monolayer blood differentiation will be to apply this technique to human ES and iPS cells to be used in clinical studies.

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