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The Role of let-7c/miR-125b/miR-99a in the Hematopoietic Development in the Human Bone Marrow

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By

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Dedication

This thesis is dedicated to my wonderful parents, who always believed in me, and my husband, without whom this would not have been possible.

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ABSTRACT

The Role of let-7c/miR-125b/miR-99a in the Hematopoietic Development in the Human Bone Marrow

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MicroRNAs (miRNAs) are a class of non-coding RNAs that regulate gene expression post-transcriptionally and in recent studies have been shown to play a key role in governing lineage in hematopoietic progenitors. Although much is known about the molecular signals that direct early hematopoiesis in mice, very little is understood about the hematopoietic process in adult human bone marrow.

The main purpose of this thesis was to identify the miRNAs that are involved in the early hematopoietic development in the human bone marrow. Microarray analysis on the hematopoietic stem cells (HSC), lymphoid primed multipotent progenitors (LMPP), and common lymphoid progenitor (CLP) from human bone marrow was used to obtain a differential miRNA expression profile.

The second part of the research was focused on validating the results using qRT-PCR on HSCs, LMPPs, and CLPs to confirm the expression of miRNAs. The results revealed microRNAs let-7c and miR-125b expression to be significantly up regulated in HSCs compared to LMPPs, CLPs and even more mature and differentiated (B-cells, T-

cells, monocytes, macrophages, Natural killer, and erythroid) cells. Furthermore, let-7c and miR-125b are a supposed to be a part of a cluster miRNA including miR-99a, we decided to assess for expression levels of all three miRNAs in all the stem, progenitor and differentiated cells to obtain a comprehensive picture of miRNA expression across the different cells. Our results revealed that let-7c, miR-125b, and miR-99 all to be highly expressed specifically in bone marrow HSCs.

Lastly, we also investigated the let-7c/miR-125b/miR-99a expression in stem and progenitor cells in human embryonic stem cells (hESCs). We found let-7c, miR-125b, and miR-99a to be indeed significantly expressed in stem like HSCs derived from hESCs as seen in bone marrow HSCs. Based on the results of our experiments, this very cell stage specific expression pattern of the miRNAs in different populations strongly suggests to us that miRNAs do play some kind of regulatory role in controlling the cell fate determination and cell lineage differentiation. It would be clinically relevant if miRNAs can be efficiently used in the expansion and maintenance of hematopoietic stem cells *in vitro* in the future.

Chapter 1: INTRODUCTION

Hematopoiesis and Lymphopoiesis

Hematopoiesis is the process in which a hematopoietic stem cell can further differentiate to give rise to all kinds of different mature blood cells in the body. A hematopoietic stem cell (HSC) has the capability to endlessly self-renew, which is the potential to divide into a new identical stem cell, or to differentiate into progenitor stem cells that can then further give rise to every kind of blood cell type in the body. Hematopoiesis occurs in a step-wise manner, in which the stem cell loses its multipotent potential as it goes through the several intermediate progenitor stages before becoming more mature and specialized cells such as B cell, T cell or Natural Killer (NK) cells with defined functions.

Developmental biology is the branch that deals with studying the mechanisms and regulatory pathways involved in the transition of the multipotent stem cells to the restricted potential, and therefore, lineage committed cells. Hematopoiesis and lymphoid commitment are dictated by a very complex network of intrinsic signals (transcription factors) and extrinsic factors (cytokines, chemokines) that coordinate the regulation of multiple gene targets to allow for differentiation of cells in the microenvironment niche, in the bone marrow.

Although much is known about the molecular signals that direct early hematopoietic development and lymphoid commitment in murine models, very little is understood about the biological cues that regulate hematopoiesis and lymphopoiesis in

the adult human bone marrow system. Most research studies on hematopoiesis and even early lymphoid commitment and development have been done on readily available cord blood but not on the human bone marrow. However, cord blood does not represent steady state post-natal hematopoietic process and significant differences in immunophenotype and function are known to exist between progenitors from cord blood when compared to bone marrow. It is also very important to study hematopoietic development and lymphoid commitment in the bone marrow as it is the site of ongoing hematopoiesis in adults.

Introduction to miRNAs: Master Regulators

MicroRNAs commonly known as miRNAs are a class of non-coding RNAs that regulate gene expression post-transcriptionally [(1)] and in recent studies have been shown to play a key role in governing lineage in hematopoietic progenitors. Mature miRNAs are short 18-22 nucleotide bases in length, non-coding RNA sequences that control gene expression. They regulate gene expression post-transcriptionally by binding to complementary sequences on the 3' UTR of the messenger RNA (mRNAs) transcripts [(2),] usually resulting in translational repression resulting in gene silencing or in some rare cases, mRNA degradation.

The longer primary transcript also referred to as the "pri-miRNA" transcript contains a stem-loop structure that is cleaved inside the nucleus by the enzyme *Drosha*[(3)] into a shorter pre-miRNA transcript (Figure 1), which is then further processed by the enzyme *Dicer* in the cytoplasm before being integrated into the RNA-induced silencing complex (RISC), resulting in translational inhibition[(4)]. Structurally, mature miRNAs are double stranded and function very much like the small interfering RNAs also referred to

as SiRNAs. In general, microRNAs are considered to play the role of negative regulators of gene expression in developmental processes of a cell.

MicroRNAs are a relatively new discovery in the field of molecular biology. The very first miRNA was identified in *Caenorhabditis elegans* in the early 1990's [(5)], but it took almost another ten years to discover and characterize a second miRNA (let-7) as a transcriptional regulator [(6)]. 25,421 different human microRNAs are listed in the miRBase registry (http://www.mirbase.org/) as of June, 2013.

These short non-coding sequences of RNA are very well conserved in eukaryotic organisms [(7)] and therefore, believed to be evolutionarily an important part of the regulatory process in the cells. However, despite the evolutionary conservation of miRNAs in organisms, they are differentially regulated and expressed in various species and play dissimilar roles in targeting and modulating [(8)] of the genes. It is indeed remarkable that while one miRNA is capable of targeting multiple messenger RNAs (mRNA), one mRNA can also be regulated by a multitude of different miRNAs; therefore, miRNAs are involved in controlling the expression of numerous related genes.

The human genome codes for 1000's of miRNAs that target multiple genes in different cell types in the body, but most of the miRNAs target genes are still unknown and unverified[(9)]. Recent studies have managed to shed some light on the mechanisms of miRNAs, which has somewhat contributed to our understanding of miRNA processing and how it affects cell functions. However, the extent of the regulation by miRNAs on gene expression in cell differentiation and development process is still largely unknown

and hence, it is necessary to properly classify and characterize the microRNAs to the specific cell type and gene function.

MicroRNAs and Disease

It has been well established from several studies over the last few years that miRNAs are differentially expressed in a wide variety of cells and tissue types and therefore, can vary among different organisms. Of note, abnormal expression levels of miRNAs have been associated mostly with various disease states, particularly cancer [10-12]. MiRNAs are also sometimes referred to as "oncomirs" as they have been found to be linked to certain kinds of cancers in the recent years. Since, miRNAs regulate gene expression by binding to messenger RNA (mRNA) and inhibiting translation of the corresponding protein, this can lead to inhibition of important proteins for example, tumor suppressor protein, inducing RISC ,which can then induce uncontrolled cell proliferation and cancer.

MicroRNAs can also affect the expression of important genes by causing histone modification of the DNA and methylation of the promoter regions [13, 14] leading to gene silencing, thus resulting in transcriptional repression and thus, loss of gene expression. Depending on the significance and function of the protein in the cell, aberrant expression or complete loss of protein can also lead to disease such as cancers.

Significance of miRNA studies and their applications

Based on what we know so far about the evolutionary conserved nature of miRNAs, they can be used as potential phylogenetic markers [15]. Measuring the level of

gene activity for different cancers can be used to understand cancer genes activity expression profile. This can then be used to pinpoint the original cancer tissue type and design a treatment plan [16]. Cancer screening assays based on miRNA profiling are being developed and used in clinical trials [17, 18].

Identification and characterization of miRNAs can be potentially used in translational research as distinct miRNA expression signatures are implicated in specific tumor classification and prognosis [19]. In addition, miRNAs are easily detectable in bodily fluids like serum and blood, and therefore, based on the expression level assays, specific miRNAs can be used as biomarkers in cancer [20, 21] for prognosis and therapeutic response to treatment.

A Recent breakthrough in reprogramming of somatic cells into induced pluripotent stem cells (iPS) [22] in the presence of the transcription factors *Oct4*, *Soc2*, *Klf4*, and *Myc* are of tremendous importance in research. They also found that *Oct4* and *Sox2* can bind to the miR-302, which plays a critical role in the reprogramming of human fibroblasts to induced pluripotent stem cell stage [23- 25] by promoting mesenchymal to epithelial differentiation. Based on these interesting findings, it is not a distant possibility that discovery of new cell-stage specific miRNAs can be used to generate induced pluripotent cells. By the same logic, it is also possible that induction of cell specific miRNAs into cells along with the important transcription factors can be used to give rise to specific hematopoietic stem or progenitor cell types, which can be used in clinical approaches in the future.

Another exciting use of miRNAs is in the expansion and maintenance of

hematopoietic stem cells *in vitro*. Hematopoietic stem cells (HSC) transplant from healthy donor or cord blood is a routine mode of treatment for various hematological diseases such as leukemia. Post irradiation of cancerous cells in conjunction with chemotherapy is commonly performed in leukemia patients before the transplantation of healthy donor hematopoietic cells. However, the limited numbers of cells tend to delay hematopoietic reconstitution in transplant patients, which in turn leaves their immunocompromised system highly susceptible to infections. One of the alternatives to overcome this hurdle would be to transplant a higher number of hematopoietic stem cells in the patients, which can lead to a rapid hematopoietic reconstitution and a speedy recovery. It would be clinically relevant if miRNAs can be efficiently used in the expansion and maintenance of hematopoietic stem cells *in vitro* in the future.

One of the changes faced by researchers is the complexity of the hematopoietic process, which is an intricate network of a combination of signaling pathways and transcription factors that play a role in a cascade of reactions that can maintain the "stemness" of cell or lead it down the path of differentiation in the hematopoietic microenvironment niche. No one single miRNA or transcription factor is alone to completely regulate and induce developmental changes in the cells.

There are few important research studies that have focused on the matter of HSC expansion and have met with some success in the recent years. It has been seen that miR-125 is involved in significant increase (eight-fold) in the number of hematopoietic stem cells [(26)]. Overexpression of miR-146a has also been implicated in HSC expansion in murine model [(27)]. In the most recent studies of the effects of miRNAs involved in

hematopoiesis, down-regulation of miR-126 has also been reported to lead to HSC quiescence and expansion [28]. However, no single miRNA has been fully validated to completely regulate or maintain HSC renewal and expansion endlessly. Due to the interaction of the signaling pathways with different regulatory factors within the intricate hematopoietic microenvironment niche, it appears likely that there may be several miRNAs in conjunction with other biological factors that tightly regulate HSC development and differentiation. Nevertheless, it is of critical significance to further investigate and characterize the miRNAs and their target genes in HSC stem and progenitor cells.

MicroRNAs and Hematopoiesis

MicroRNAs are involved in various cellular developmental processes such as cell differentiation and even apoptosis, which makes them very interesting candidates for research purposes. Several studies have been done on miRNAs in the last few years to identify the novel miRNAs and investigate the effects of their expression on the hematopoietic stem and progenitor cells. Most of the recent studies have suggested that up-regulation of certain miRNAs at stage-specific stages of hematopoietic development may play a critical role in their differentiation and lineage specification. However, most of these studies have been done on murine models and cord blood in humans. There are differences in mice and human cell markers and therefore, researchers are faced with several challenges when comparing miRNA signature profiles in the two different species [29].

Most recent studies have identified various miRNAs involved in different levels of hematopoietic stages of blood cell stage developmental process. For example, expression of miR-150 has been shown to drive megakaryocyte-erythroid progenitors (MEPs) towards the myeloid differentiation pathway [30]. miR-451, miR-16, and miR-144 have been demonstrated to be positive regulators, while miR-50, miR-155, miR-122, and miR-223 have been found to be the negative regulators of erythropoiesis (development of red blood cells) [31-34]. Up-regulation of miR-424 has been implicated to play an integral role in the monocyte/macrophage differentiation in the myeloid pathway [35, 36]. Collectively, these findings provide strong evidence to support the theory that miRNAs do control various aspects of hematopoietic stem and progenitor cells and are indeed key players in the cell fate determination and lineage commitment. MicroRNAs have now been recognized to play an important role in lineage commitment at the level of restricted hematopoietic progenitors [37].

Our lab recently identified a novel hematopoietic progenitor in human bone marrow, defined by the immunophenotype $CD34^{+}Lin^{-}CD10^{-}CD45RA^{+}CD62L^{hi}$ [38]. This $CD10^{-}CD62L^{hi}$ population (aka lymphoid-primed multipotent progenitors or LMPPs) exhibited T, B, NK, and monocytic potential, but lacked erythroid potential (similar to the murine LMPP). Of note, this population could give rise to the $CD34^{-}Lin^{-}CD10^{+}$ common lymphoid progenitor (CLP) phenotype *in vitro*, and microarray analysis showed a global gene expression profile intermediate between the earliest hematopoietic stem cell CD34+CD38-Lin-CD45RA- (HSC) and the later common lymphoid progenitor (CLP), suggesting that it represents the earliest known stage of lymphoid differentiation in the human bone marrow (Figure 2).

In order to obtain a comprehensive picture of the lineage programs present at this earliest stage of hematopoiesis and lymphoid differentiation, we decided to investigate the miRNAs involved in the hematopoietic stem cells (HSCs) and in later progenitor stages. Furthermore, we also investigated the differential expression of miRNAs in our stem and progenitor cell populations (HSCs, LMPPs, and CLPs) to the more mature, specialized functional cells such as B (CD19+), T (CD10+), Natural Killer (NK) CD56+, monocytes/macrophages (CD14+/CD15+), and erythroid cells (CD235a+) for any significant differences in expression pattern among different cell types (Figure 2).

Human embryonic stem cells (hESC) are also considered to be a valuable source that can be used to manufacture any type of cell *ex vivo*. It is well known that a hematopoietic stem cell niche is comprised of an intricate cellular microenvironment and its structure plays an important role in the hematopoietic development of cells. Some studies have highlighted the connection between HSCs and MSCs, mesenchymal stromal cells in the maintenance and regulation of stem cell function [(39-41)]. MSCs possess the potential to give rise to various cell types such as adipocytes, osteoblasts, and chondrocytes and miRNAs are believed to be involved in the proliferation and differentiation of MSCs [(42)].

One of the projects in our lab focuses on using the hESCs to generate erythroid progenitors in order to manufacture red blood cells (RBC). hESCs, SSEA4+ (Stage Specific Embryonic Antigen4) are cultured and are used to generate human embryonic mesodermal progenitor cells (hEMP) CD326-/CD56+, which in turn give rise to mesenchyme (multipotent stromal cells). MSCs give rise to the earliest, primitive

hematopoietic stem cells (Hem) CD33-/CD38-/CD34+/CD43+ lacking myeloid potential, then hematopoietic stem cells (HSC) CD34+, various hematopoietic progenitors (MEP) CD43+/CD41a+/CD42a+/GlyA+ as well as mature and differentiated cells. Transduction of various stem and progenitor cells derived from hESCs with F36 MPL vector (fusion protein) in the presence of synthetic diffusible ligand, CID (chemical inducer of dimerization) results in significant expansion of CD34+ HSC cells with multi-lineage potential. Hence, we also decided to explore the presence or absence of the microRNAs of interest in the earliest embryonic stem cells, primitive hematopoietic stem cells, HSC like stem cells, stem cells transduced with erythroid driven vector MPL in presence of erythropoietin factor (EPO), CID to improve erythroid potential or untransduced; hematopoietic progenitors (MEPs aka Megakaryocyte-erythroid Progenitors) and differentiated erythroid cells (Figure 3).

We were really interested to find out if our miRNAs candidates were also present and expressed in culture derived hematopoietic stem cells and if so, at how early a hematopoietic stage? Would we observe a similar pattern of expression as we had seen with our bone marrow experiments or are miRNAs just present in adult stages of human hematopoietic development? The ultimate goal was to pinpoint the specific microRNAs expressed significantly only at the hematopoietic stem cell stage but not at other progenitor and differentiated cell stages and vice versa.

We hypothesized that by investigating the various stages of different hematopoietic cell subpopulations in the human bone marrow, we will be able to identify a distinct subset of stage-specific expressed miRNAs that regulate transcription factors

and thus, maintain hematopoietic stem cells and regulate lineage commitment in hematopoietic cells. Comparison of differential expression data of miRNAs of interest across the different stem, progenitor and differentiated cell populations in the bone marrow and hESCs helped us recognize a specific miRNA signature in the hematopoietic stem cells.

MicroRNA Profiling: Techniques

One of the problems in miRNA profile studies is that miRNAs are expressed at low levels in cells. Therefore, it was important for our research to use fresh bone marrow samples and sort the different stem (HSCs) and progenitor cell (LMPP and CLP) populations on stringent gating strategy in addition to a combination of accepted and well known cell surface markers to distinguish different cell populations. The TRIzol based extraction method works very well to efficiently isolate total RNA (less than ~ 200 bps in size) from cells while keeping it intact. Even though miRNAs can be challenging subjects due to their small size, there are studies that emphasize the benefits of small miRNA size [(43)] as they are highly expressed in cells and with proper technique, can be easily processed without any degradation.

Microarray analysis is the technology that uses a chip which comes with thousands of prepared probes to which your sample can hybridize. The intensity of the hybridization measures the level of gene expression of your sample. This data can be used as a screen to profile and quantify the presence or absence of your genes in the specific cell population sample. However, there are some limitations to this method and therefore, deep sequencing or next generation sequencing (NGS) of the whole

transcriptome of the bone marrow HSCs, LMPPs, and CLP cell populations in addition to microarray array provides a deeper insight into the overall differential expression pattern of miRNAs and also the discovery of any novel miRNAs and genes.

Combination of microarray data and the gene expression profile of different cell populations can provide a clearer and distinct picture of miRNA genes expression profile in any specific population at a time. The data from these analyses can also be used to generate heat maps showing differential expression of miRNAs across the different stem and progenitor populations for comparison purposes. In addition, the data can be used to create hierarchical clustering dendrograms trees, which can then assess the closely associated miRNAs that may be co-regulators and play a role in maintaining specific stem and progenitor cells.

The gold standard for quantifying and assessing levels of RNA transcripts [(44)] for any nucleic acids in specific cell populations is the Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) and we used this technology to quantify the expression levels of miRNAs in different stem and progenitor populations. Based on the qRT-PCR data generated by the miRNA assays, we were able to categorize miRNAs that are highly expressed in HSCs (Hematopoietic Stem cells) and down-regulated or absent in the other three LMPPs (Lymphoid-Primed Multipotent Progenitors), and CLPs (Common Lymphoid Progenitors) populations. Similarly, we also found some miRNAs to be up-regulated in LMPPs and up-regulated in CLPs and down-regulated in the others cell subpopulations. The variance in expression levels in different cell types suggests to us that these miRNAs do play an important role in maintaining the “stemness” of the

early stem cells and in their differentiation to more mature cells in later developmental stages of hematopoiesis.

The future aim of this study is to carry out *in vitro* and *in vivo* studies to corroborate our findings. MicroRNAs let-7c, miR-125b, and miR-99 will be used to transduce specific stem and progenitor cells in order to investigate and characterize the functional potential by cell specific colony forming unit assays (CFUs). In addition, lentiviral vectors carrying the selected miRNAs will be used to infect stem and progenitor cells to grow in culture, which will then be transplanted into mice for further study.

To determine if expression of specific miRNA affects differentiation, flow cytometry will be used to evaluate frequency of various mature lymphoid populations (B, T, and NK cells) compared to empty vector transduced and untransduced control cells. The data from the whole transcriptome sequencing (RNA-sequencing) of bone marrow HSCs, LMPPs, and CLP populations will further help us to understand the lineage programs (pluripotency and self-renewal genes) present at the earliest stages of hematopoietic development. Furthermore, the RNA seq data will help define novel molecular signals and pathways involved in the maintenance and regulation of HSCs in the human bone marrow.

CHAPTER 2: MATERIALS & METHODS

Isolation of stem cells from Human Bone Marrow

Normal human bone marrow samples were obtained from healthy donors via the UCLA Pathology Tissue Core, Cincinnati Children's Hospital, or ALLCELLS according to guidelines approved by UCLA Institutional Review Board. Fresh BM samples were Ficoll-Paque (Sigma Aldrich) processed to remove red blood cells and isolate different white blood cells (MNCs). CD34+ cells were further isolated from other white blood cell populations by staining cells with a magnetically labeled CD34 antibody (MACS) sorting, and running it through a magnetically charged column to collect the CD34+ hematopoietic stem cells (Milteyni Biotech).

FACS: Fluorescent Activated Cell sorting

The CD34+ enriched cell fraction was then further isolated based on cell surface markers specific to various stem and lymphoid progenitor cell populations. CD34+ cells were incubated and immunostained using the combinations of the following anti-human specific monoclonal antibodies CD34-APC-Cy7 (581), CD38-APC (H1T2) , CD62L-PE (DREG-56), CD45RA-PerCP Cy5.5 (HI100), CD10-PE-Cy7 (HI10a) (Biolegend). Lineage depletion monoclonal antibodies for cell sorting used were CD3-FITC, CD235a-FITC (aka Glycophorin A) (Beckman Coulter), CD14-FITC, CD15-FITC, CD19-FITC, CD56-FITC, and CD57-FITC (Biolegend). 4'6-diamidino-2-phenyllindole (DAPI) was used as a viability dye to sort for live versus dead cells. Negative gates were based on a no-antibody control cell sample. Additional analysis for further studies used to sort for

differentiated lymphoid and myeloid cell populations were as follows: CD3-APC-Cy7 (HIT3a), CD14-APC (HCD14), CD15-APC, CD19-PeCy7, CD56-PE, and CD235a-FITC (Biolegend). Cells were sorted and isolated using FACSAria (355, 405, 488, 561, and 633 nm lasers) (BD Immunocytometry Systems).

The cells collected from fluorescent activated cell sorting (FACS) were HSCs or hematopoietic stem cells (CD34+/CD38-/CD45RA-/Lin-), LMPPs or lymphoid-primed Multipotent Progenitors (CD34+/CD45RA+/Lin-/CD10-/CD62L++), CLPs or common lymphoid progenitors (CD34+/Lin-/CD10+/CD19-) as seen in (Figure 4), and more differentiated populations, T-cells (CD3+), monocytes and granulocytes (CD14+/CD15+), B cells (CD19+), Neutrophils (CD56+), and also Erythroid cells (CD235a+) as seen in (Figure 5).

RNA Extraction

The individual cell subpopulations collected were lysed with TRIzol reagent (Cat#15596-026) (Ambion), which helps preserve RNAs that are less than 200 bases. RNA collected was then phenol-chloroform extracted from these cell lysates and further purified to remove impurities with miRNeasy Microkit (Qiagen). RNA quantification was done with Quant-iT RiboGreen RNA Assay Kit (Cat#R11490) (Life Technologies).

cDNA library preparation for Next Generation Sequencing

Ovation RNA-Seq System V2 (Nugen) was used to prepare and amplify cDNA from total RNA extracted from HSCs, LMPPs, and CLPs. The cDNA was then sheared to the appropriate size (~350bp) using Covaris M220 Focused UltraSonicator at UCLA Core Facility for library Next Generation Sequencing (NGS). The fragmented cDNA libraries

of the HSC, LMPP, and CLP cell populations were ligated into a NGS library using reagents and adaptors (8 unique barcoded adaptors) with Encore Rapid Library System (Nugen) for multiplex sequencing. The resulting cDNA adaptor tagged library was quantified using KAPA Quantification System (Cat#KK4835) (Illumina) and sent for next generation sequencing to the UCLA Genotyping and Sequencing Core facility (GenoSeq).

cDNA libraries for Microarray Profiling

RNA was extracted from two biological bone marrow samples with miRNeasy Microkit (Qiagen) for each of the three stem and progenitor populations (HSCs, LMPPs, and CLPs). Each RNA sample was labeled using miRCURY LNA Hi-Power labeling kit and hybridized to miRCURY LNA 6th gen microRNA Array chip (pre-printed with 1900 microRNAs LNA probes) according to the protocol. The data generated from the microarray hybridization was used to analyze microRNA expression profile in the HSC, LMPP, and CLP cell populations.

Microarray Analysis

RMA or Robust Multichip Average (Affymetrix) was used for background correction, quantile normalization and median polish method was used for probe-level summarization of miRNA gene expression intensities. Hierarchal clustering using Spearman rank correlation (distance metric) and average linkage method was used to perform gene cluster analysis. LIMMA, a linear model of analysis (R/bioconductor software package) was used to assess differential expression of microRNAs across all the different human bone marrow subpopulations (HSCs, LMPPs, and CLPs). The

expression values in fold increase of +/-2.0 and p-value of <0.05 was used to discern a distinct pattern of up-regulated and down-regulated microRNAs in pairwise comparison among the hematopoietic subpopulations across the biological replicates (sample size, n=2) on which criteria, the heat maps were generated. For drawing of the heat maps, Cluster 3.0 (Clustering) and Java Tree View (dendrograms, heat maps) software was used.

Quantitative PCR Analysis

Based on the data generated from this analysis, the miRNAs of interest were selected for quantification using qPCR. Post FACSAria isolation of all the different cell populations, total RNA was extracted using Trizol and miRNeasy Microkit (Qiagen). Taqman miRNA Reverse Transcription kit (Applied Biosystems) and Superscript III first strand system for RT-PCR kit (Invitrogen) was used to synthesize miRNA specific cDNA from the total RNA extracted from the HSCs, LMPPs, and CLPs and differentiated B, T, NK, monocytes, granulocytes, and erythroid cells. The pre-amplification and qPCR primers for the specific miRNAs were ordered through Taqman (Life Technologies). See Table (Supplementary Figure 1) for a list of Taqman miRNA primer probes used for all the qPCR experiments. Reactions were carried out in technical and biological triplicates using 96-well plates on ABI 7900 (Applied Biosystems). StepOne Software V2.3 (Applied Biosystems) was used to analyze the experiments, and qPCR expression of the candidate genes were normalized to the geometric mean of reference target genes or housekeeping genes (RNU44 and RNU48) using the ΔΔCt method.

Functional Analysis

The next phase of this study will be to investigate the effect of miRNA expression on various stem and lymphoid progenitor populations both *in vitro* and *in vivo*. Stem and lymphoid progenitors will be infected with vectors carrying the specific miRNAs of interest and grown in culture or transplanted into mice. To determine if the expression of specific miRNA affects differentiation, flow cytometry will be used to evaluate frequency of various mature lymphoid populations (B, T, and NK cells) compared to empty vector transduced and untransduced control cells.

CHAPTER 3: RESULTS

Differential expression of microRNAs in the stem and progenitor cells in the human bone marrow

The data from the microarray hybridization chip from our human bone marrow samples was analyzed and used to measure differential expression of miRNAs. The .gpr files from the microarray chip were read for the red channel (Hye3 dye) and the values were then used to calculate the median foreground and background. RMA or Robust Multichip Average was used for background correction, quantile normalization and median polish method was used for probe-level summarization of miRNA gene expression intensities.

LIMMA, a linear model of analysis was used to assess differential expression of miRNAs across all the different human bone marrow subpopulations (HSCs, LMPPs, and CLPs). The expression values in fold increase of +/-2.0 and p value of <0.05 was used to discern a distinct pattern of up-regulated and down-regulated miRNAs in pairwise comparison among the hematopoietic subpopulations across the biological replicates (sample size, n=2) in technical triplicates. Based on these two criteria, we were able to identify several miRNAs that appear to be significantly up-regulated or down-regulated in specific cell populations.

The results from Microarray data analysis indicated 29 miRNAs to be up regulated in the very early hematopoietic stem cell populations CD34+/CD38-/Lin- (HSCs), 10 miRNAs up regulated in the Lymphoid-primed Multipotent progenitor population CD34-/CD38-/CD10-/CD62Lhi (LMPPs), and 9 miRNAs up regulated in the Common

Lymphoid Progenitors CD34-/CD38-/CD10+ (CLPs) with at least 2.0 fold increase in expression and a p value of <0.05 in pairwise comparison of the biological samples (Figure 6).

MiR-10a, miR-125b, miR-223-3p, miR-126a, miR-24-3p, miR-4288, miR-29a, miR-580, miR-654-3p, miR-3147, miR-196-5p, miR-146a-5p, let-7c, miR-155, and miR-99a among others appeared to be highly expressed in the hematopoietic stem cells (HSCs) in the human bone marrow samples based on the microarray analysis. In Lymphoid primed multipotent progenitors or LMPPs, we found miR-1288, miR-129-3p, miR-643, miR-19a-5p, miR-3135a, miR4446-5p, and miR-611 to be highly expressed as seen in (Figure 6). In addition, miR-1248, miR-4322, miR-15a-5p, miR-16-5p, miR-4301, miR-135b-3p, and miR-574-5p to be expressed at a significantly higher level in the common lymphoid progenitor or CLPs.

In order to validate the results of the microarray analysis, we performed qRT-PCR with the specific miRNA stem-loop primer assays (Taqman) to quantitate miRNA expression analysis in specific stem, progenitor, and differentiated cell populations. RNU44 and RNU48 (housekeeping genes) were used as the endogenous control to normalize and quantify miRNA relative expression in all stem and cell progenitor populations across all the different bone marrow samples.

Unique pattern of Let-7c/miR-125b/miR-99a Expression in HSCs from Human Bone Marrow

Based on the qRT-PCR data analysis, we found that let-7c to be highly expressed in all the hematopoietic stem cells (biological samples, n=3) by ±2.5 fold compared to

LMPPs and CLPs (Figure 7). MicroRNA, miR-125b was also found to be significantly up regulated in the HSCs (±25 fold) compared to LMPPs and CLPs (Figure 8). In the second set of experiments when comparing to expression in differentiated cells, let-7c was once again seen to be significantly expressed in HSCs specifically at levels higher than LMPPs, CLPs, B-cells, T-cells, NK cells, Macrophage/Monocytes, and erythroid cells (Figure 9). While comparing expressions of miR-125b in differentiated cells, the results were again consistent with higher expression in HSCs compared to progenitors and differentiated cells (Figure 10). Another miRNA of interest, miR-99a (part of the let-7c, miR-125b, and miR-99a microRNA cluster) was also expressed at ±12 fold in HSCs (Figure 11) compared to LMPPs, CLPs and all other differentiated hematopoietic cells. Overall, the expression of all three miRNAs in the LMPPs, CLPs, B-cells, T-cells, Macrophages/Monocytes, NK cells, and erythroid cells were either very low or completely absent.

Let-7c/miR-125b/miR-99a also highly expressed in the hematopoietic stem cells derived from human embryonic stem cells

The highest expression of let-7c, miR-125b, and miR-99a was observed in the earliest primitive stem cells of hematopoietic nature (hem-MPL or hem+MPL) derived from human embryonic stem cells (hESCs) and hematopoietic stem cells (HSC-MPL or HSC+MPL) when compared to the early non-hematopoietic hESCs (-MPL or +MPL), the human embryonic mesodermal progenitors (hEMP-MPL or hEMP+MPL) and also in the more mature, Megakaryocyte-Erythroid progenitors (Figures 12, 13, and 14). In addition, we observed let-7c, miR-125b and miR-99a to also be down-regulated or absent in the non-hematopoietic hESCs prior to their transformation to hematopoietic cells and

tapering down of the miR expression as the stem cells differentiated into the mature hematopoietic progenitor stage, MEP. Moreover, the expression levels of let-7c, miR-125b, and miR-99a were significantly lower even in Hem and HSC when transduced with erythroid driven vector MPL in conjunction with erythropoietin factors, EPO and CID, which strongly indicated the importance of the role of miRNAs let-7c, miR-125b, and miR-99a at the stem cell level in hematopoietic development respectively(Figures 12, 13, and 14) .

Let-7 family microRNA Expression Profile

We wanted to further investigate the microRNAs, especially the much conserved group of miRNAs, let-7 miRNA family (let-7a, let-7b, let-7c, let-7d, and let-7g) including miR-99a as a cluster that are up regulated in hematopoietic stem cells compared to other LMPP and CLP progenitor populations as seen from the heat maps generated by the microarray analysis. Additionally, we also decided to sort and investigate the more mature (CD34-), differentiated and functional cells CD3+ (T-cells), CD14/CD14+ (Monocytes/Macrophages), CD19+ (B-cells), CD56+ (NK or natural killer cells), and CD235a+ aka GlycophorinA+ or GlyA+ (erythroid cells) in order to identify the specific let-7 miRNA involved with various stage specific stem and progenitor cells.

The results from our qRT-PCR experiments let-7a and let-7b were observed to be up regulated in HSCs when compared to LMPPs and CLPs but down regulated in the differentiated B, T, NK, monocytes, macrophages and erythroid cells (Figures 15a & b). Furthermore, let-7d and let-7g were expressed in all kinds of stem, progenitor and differentiated cells suggesting to us that these miRNAs are not specific to any certain

stem or progenitor cell population or perhaps may be involved in targeting more than one cell population (Figures 15c & d).

Notwithstanding the expression of let-7a and let-7b expression in HSCs, let-7c was highly expressed in hematopoietic stem cells (HSCs) when compared to LMPP and CLP progenitors. In addition, it was also observed to be down regulated in the mature T, NK, monocytes, macrophages, and erythroid cells and not expressed at all in the differentiated B cells. Similar pattern of high expression in HSCs and low expression or no expression at all was also seen in miR-99a. Collectively, this data strongly indicated that let-7c may be one of the miRNAs involved in the hematopoietic stem cell maintenance at the earliest stage in combination with miR-99a and miR-125b along with other transcription factors.

Quantitative expression analysis of miRNAs in hematopoietic stem, progenitor, and differentiated cells

Other HSC microRNAs based on microarray analysis, such as miR-29-3p was expressed at ±6.8 fold higher levels (supplementary figure 2); miR-24-3p was expressed at ±5 fold higher levels (supplementary figure 3), and miR-654-3p was also observed to be expressed at ±4 fold higher levels when compared to the LMPPs and CLP cell populations (supplementary figure 4**)**. However, miR-146a was observed to be highly expressed in HSCs but also in one CLP sample and there was also some expression expressed in LMPPs (supplementary figure 5), while miR-223-3p was highly expressed in HSCs (±16 fold), but at similar levels in LMPPs and at much lower levels in CLPs (supplementary figure 6). Furthermore, miR-10a was highly expressed in HSCs compared to LMPPs and CLPs but only in one bone marrow sample (data not shown).

Certain miRNAs such as miR-580, miR-3147, and miR-4288 were not expressed at all in any of the three stem and progenitor populations in our experiments (data not shown).

LMPP specific miRNAs selected for quantification, miR-643, miR-1288, and miR-129-3p were not expressed in lymphoid primed progenitor cell populations as expected, but we also did not observe any amplification product in the stem (HSC) and progenitor (CLP) cell populations tested for expression (data not shown). CLP specific miR-4322 was seen to be expressed at 1.0 to 5.5 fold levels higher than seen in the hematopoietic stem cells (HSC) and the lymphoid primed progenitor (LMPP) cells (supplemental figure 7). Although miR-135b was also found to be highly expressed in CLPs, there was also high expression seen in LMPPs from one bone marrow sample (supplemental figure 8). However, miR-1248 was not expressed in the CLPs as expected or the other stem HSCs or the CLP progenitor cells (data not shown).

Based on cumulative qRT-PCR results of all the miRNAs and their expression across the stem, progenitors, and differentiated cells, we were able to observe a pattern of expression in let-7c, miR-125b, and miR-99a that was unique and specific to the hematopoietic stem cells, HSCs in the different bone marrow samples. Furthermore, the emergence of a similar pattern of increased expression of let-7c, miR-125b, and miR-99a in early hematopoietic stem cells derived from hESCs and reduction in expression in the megakaryocyte-erythroid progenitors further demonstrated to us that these three miRNAs are expressed at the specific stem cell stage and may play a vital role in hematopoietic stem cell maintenance.

CHAPTER 4: DISCUSSION

Our study has successfully used the microarray analysis technology to identify novel microRNAs expressed in the different hematopoietic stem and progenitor cell populations in the human bone marrow. The results of the quantitative PCR experiments further provide evidence to support our hypothesis that microRNAs are indeed important regulatory elements that probably play a critical role in stage-specific hematopoietic development. Based on the differential expression pattern of the specific miRNAs observed in the particular stem and progenitor cells, it is very likely that these non-coding RNAs are involved in the developmental and differentiation pathway of hematopoietic developmental process.

The up regulation of miRNA let-7c expression in the very specific Hematopoietic stem cells (HSC) compared to all the lymphoid-primed progenitor (LMPP), the common lymphoid progenitor (CLP) in addition to all five differentiated mature T, B, NK, monocytes, macrophages, and erythroid cells strongly suggests to us that the presence of microRNA let-7c is important at the HSC stage and its down-regulation may contribute to differentiation of stem cells to progenitors.

Role of Let-7c/miR-125bmiR-99a in Hematopoiesis

Let-7 is also an evolutionary conserved miRNA that appears to govern cell proliferation and differentiation [(44)] in several species. It has also been discovered that let-7 is highly expressed in normal tissues compared to cancerous cells in lung cancer [(45)]. Of note, most of the studies on let-7 expression mention just the miRNA “let-7” even though

there are several members of the let-7 miRNA family involved in different cellular processes. Based on previous findings and our own data, we think it is likely that we have found a new member of the let-7 family, let-7c specifically to be involved at the hematopoietic stem cell stage. No other let-7 family miRNAs (7a, 7b, 7d, or 7g) were expressed in a cell specific pattern to be considered as a cell stage specific miRNA of interest besides let-7c.

All the other let-7 family miRNAs were seen to be expressed at more or less similar levels in all the stem, progenitor and differentiated cell levels, which may very well be due to the fact that these miRNAs differ from each other just by a single nucleotide [(45)] that makes it hard to detect individual miRNAs. Remarkably, in spite of this dilemma among the various let-7 family miRNAs, let-7c expression was specific and seen to be significantly higher only in the stem cells repeatedly in our experiments. Furthermore, miR-99a expression level in HSCs was found to be similar to let-7c and therefore, we suggest that let-7c and miR-99a may be co-regulator miRNAs involved at the earliest stem level stage of hematopoietic development.

Another miRNA of interest we identified is miR-125b, which we observed to be ±25-fold up-regulated in HSCs compared to LMPPs and CLPs. The highly expressed miR-125b as seen in HSCs suggests to us that it may also play an important role in hematopoietic developmental stage in the human bone marrow. Interestingly, overexpression of miR-125b has been also found to cause enhanced hematopoietic engraftment in mice [(46)], which indicates its role in modulating hematopoietic output.

It was very exciting to observe that let-7c was highly expressed in stem cells of the hematopoietic nature compared to all other stem or progenitor cells derived from human embryonic stem cells, hESCs. We found similar expression of miR-125b, and miR-99a to be up-regulated specifically in the earliest hematopoietic stem cells (hem) CD34+/CD33-/CD38-/CD43+, hematopoietic stem cells (HSC) CD34+ compared to all other hESCs. Moreover, let-7c, miR-125b, and miR-99a seemed to be either expressed at very low levels or completely absent in the hESCs and human embryonic mesodermal progenitors (hEMP) when compared to hematopoietic stem cells. Subsequently, we also found that all the above mentioned miRs levels appeared to drop as the stem cells progressed and differentiated into more mature progenitor stages.

Interestingly, we also noted that the levels of let-7c, miR-125b, and miR-99a seemed to lower even in hematopoietic stem cells transduced with erythroid driven MPL vector in presence of EPO and or CID, which strongly suggests that as the cells are skewed towards erythroid potential, the miRs expression is down regulated. Collectively, all the results strongly support the theory that expression of let-7c, miR-125b, and miR-99a are indeed required at the stem level in hematopoietic development and absence or down regulation of these miRNAs can signal the start of the differentiation process of stem cells to the next progenitor stage in combination with other transcription factors.

Analysis of microRNAs at different stem and progenitor stages

We also found miR-29-3p to be highly expressed 6.8 fold in HSCs relative to LMPPs and CLPs. Exogenous expression of miR-29-3p in murine HSCs and progenitors has been seen to result in skewing lymphoid stem cells towards myeloid lineage and

enhanced self-renewal potential [47]. It would be curious in the future to see if it has a similar pattern of expression in human hematopoietic stem and progenitors in the human bone marrow as well. MiR-10a was another miRNA of interest that was also observed to be highly up-regulated but only in one HSC sample and therefore the experiments need to be repeated for additional validation.

Our results showed miR-223-3p to be expressed 16 fold higher in HSCs compared to CLPs. However, we also found similarly high levels of expression in LMPPs as seen in HSCs, which indicates that it might not be HSC specific, even though some studies have suggested that miR-223 is hematopoietic specific and plays a functional role in the myeloid lineage development [48, 49]. It has also been proposed to have a functional role in monocyte and erythroid proliferation and differentiation at progenitor and precursor levels [49, 50]. Therefore, it is important to fully investigate miR-223-3p expression pattern in the differentiated CD14+/CD15+ monocytes/macrophages, and CD235a+ (GlycophorinA+) erythroid cells in the human bone marrow to perhaps discern a more functional role in hematopoietic capacity in the future.

There was no significant difference observed in the expression of miR-146a-5p between HSCs, LMPPs, and CLPs. Other studies have found miR-146 to be involved in NF-kB activation and the immune response system pathway [51]. It has been indicated to play a role in T-lymphocyte differentiation and T-cell expression and our expression data also shows miR-146a-5p to be highly expressed in CLPs or the common lymphoid progenitors. Nonetheless, it will be exciting to investigate the expression level of miR-146a in mature differentiated cells, especially CD3+ T-cells in experiments specifically

focused on lymphoid development.

MicroRNA-4322 was observed to be significantly expressed in CLPs in our qRT-PCR experiments compared to HSCs and LMPPs (supplementary figure 7). However, there are no studies or experiments suggesting any functional role of miR-4322 in any hematopoietic process and therefore, it is essential to do further experiments to characterize this newly discovered CLP miRNA in detail. In our experiments, miR-135b was also up-regulated in the CLPs compared to LMPPs (supplementary figure 8), especially HSCs. Previous studies have found miR-135 to be implicated in kinase linked oncogenic activities [(52)] but no studies have linked to its role in hematopoietic development or differentiation yet. Therefore, miR-4322 and miR-135b are newly discovered miRNAs that will need to be further studied to fully investigate their distinct role in hematopoietic progenitor differentiation pathways.

Lymphoid specific miR-19a was seen to be up regulated only in lymphoid progenitors, LMPPs as expected but not in HSCs and CLPs albeit in only one sample. Lower level of expression may be due to the low number of LMPPs in the human bone marrow samples. Regardless of the expression level, it is interesting to note that miR-19a is a part of the 6 miRNA cluster (miR-17, miR-18, miR-19, miR-20, miR-92, and miR-106), which are indicated to be oncogenes that play a role in proliferation, inhibition of apoptosis, and inducing tumor angiogenesis [(53)]. However, there is much more work to be done in order to fully comprehend the role of miR-19a expression in all stem and progenitor in addition to differentiated cells and also perform functional analysis for further clarification.

Future of microRNAs

One of the major problems frequently faced by researchers in studying microRNAs are that they are differentially expressed in various tissues and cells and can have diverse targets and altered functions in unlike tissues. In addition, a lot of miRNAs have previously been predicted based on computational algorithms but not have been identified in any specific cells or tissues or even functionally validated for that matter. However, the discovery of miRNAs and their recognition as key regulators in modulating developmental and differentiation process has revolutionized the field of molecular biology and generated several studies.

The identification and classification of specific miRNAs involved in various tissues and cells can very well be used to compare the differences between the normal and diseased states, which can then be used for diagnostic and therapeutic purposes in the future. In fact, there are several miRNAs that have been identified to play a role in various diseases and have been patented as therapeutic targets. On the other hand, it would be of paramount relevance clinically if miRNAs along with other important regulatory factors can be used to generate a vast supply of hematopoietic stem cells *in vitro* that can be then used successfully in HSC transplantation in patients.

CHAPTER 5: CONCLUSION

To summarize our results, we have for the first time identified and presented a novel panel of miRNAs (let-7c, miR-99a, and miR-125b) involved in the hematopoietic development at stem (HSC) cell stage in human bone marrow. In conclusion, the preliminary data from the microarray analysis and qRT-PCR data from miRNAs in the stem and progenitor cells has helped us to identify a very distinctive miRNA expression signature that will be used for further characterization of miRNAs in hematopoietic development and differentiation.

Our data and results strongly supports the hypothesis that microRNAs let-7c/miR-125b/miR-99a are important key players and may regulate the stem cells at the earliest stage of the hematopoietic development. Based on many studies, it has been established that all of the mature blood cells are produced by the activation of specific differentiation programs in the self-maintaining stem cells [(54)]. Hence, we propose that it is very likely that the specific expression of these miRNAs along with other biological signals-extrinsic and intrinsic, may be involved in initiating the repression of the genes responsible for the differentiation pathway including the activation of genes critical for the self-renewal or maintenance of stem cells (Figure 16).

Additional experiments and most importantly, functional assays of the selected miRNAs of interest are required to further elucidate the regulatory mechanisms in hematopoietic development in the human bone marrow. Differential expression of microRNAs in the very specific early stem or the later progenitor stages of hematopoiesis at the very least supports the hypothesis that they are very important modulators along

with other transcription factors and cytokines, involved in a very complex and tightly controlled biological process. Further comprehension of the different miRNAs involved in various cell types can be a very powerful approach to associate specific phenotypes for comparison in normal versus disease states. Most importantly, understanding the comprehensive picture of the role of specific miRNAs involved at distinct stages of hematopoietic development can be used *in vitro* cultural expansion on HSCs, providing a continuous, inexhaustible source of stem cells for use in transplants, which is a promising idea in the field of stem cell research.

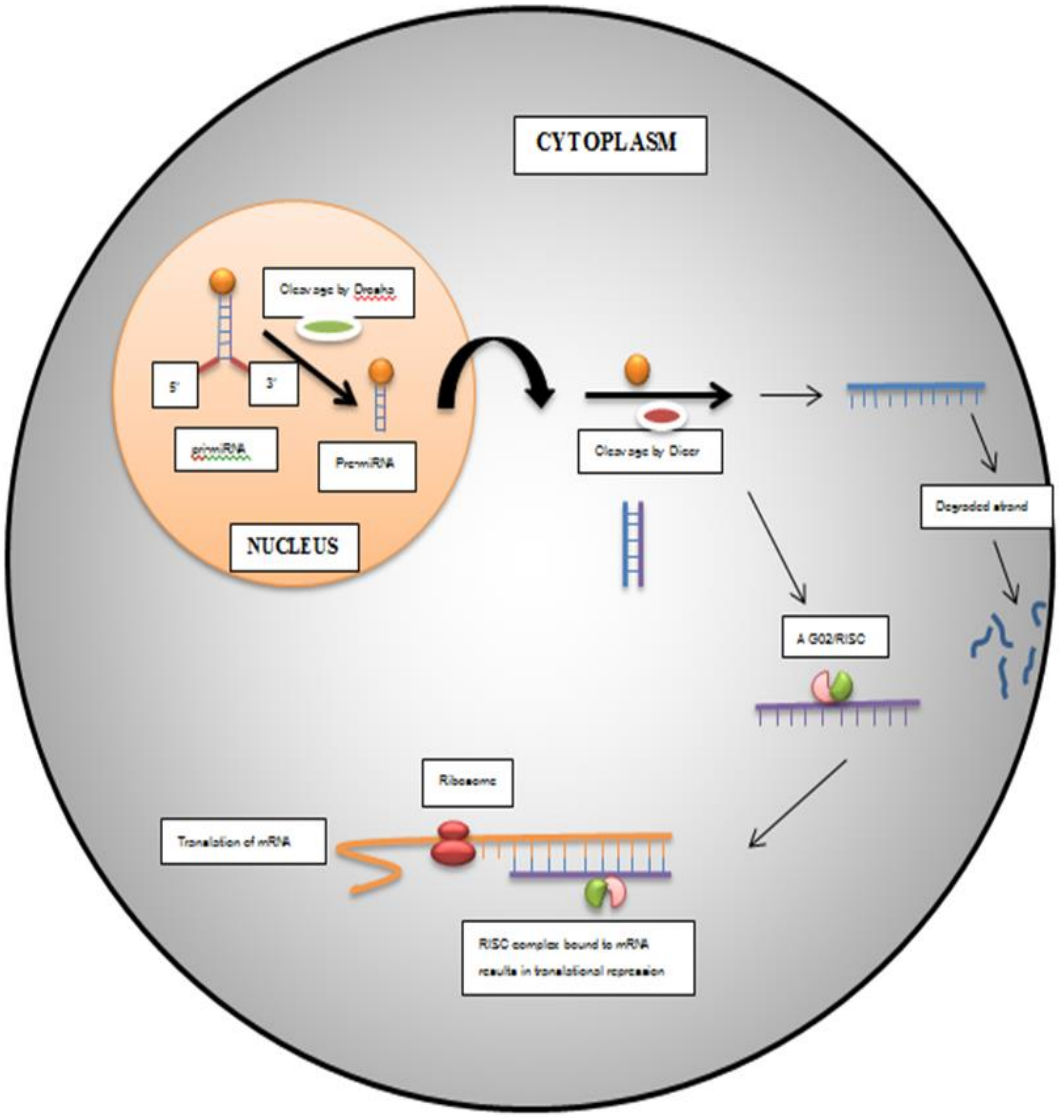

Figure 1. Diagram of microRNA biogenesis pathway

The longer primary miRNA transcript (aka Pri-miRNA) is cleaved by the enzyme *Drosha* in the nucleus of the cell. The newly cleaved, shorter transcript "pre-miRNA" is exported out of the nucleus with the help of protein Exportin-5. In the cytoplasm, pre-miRNA is further processed and cleaved yet again into a mature miRNA by the enzyme D*icer* before being integrated into the RNA-induced silencing complex (RISC), leading to translational repression of protein or mRNA degradation.

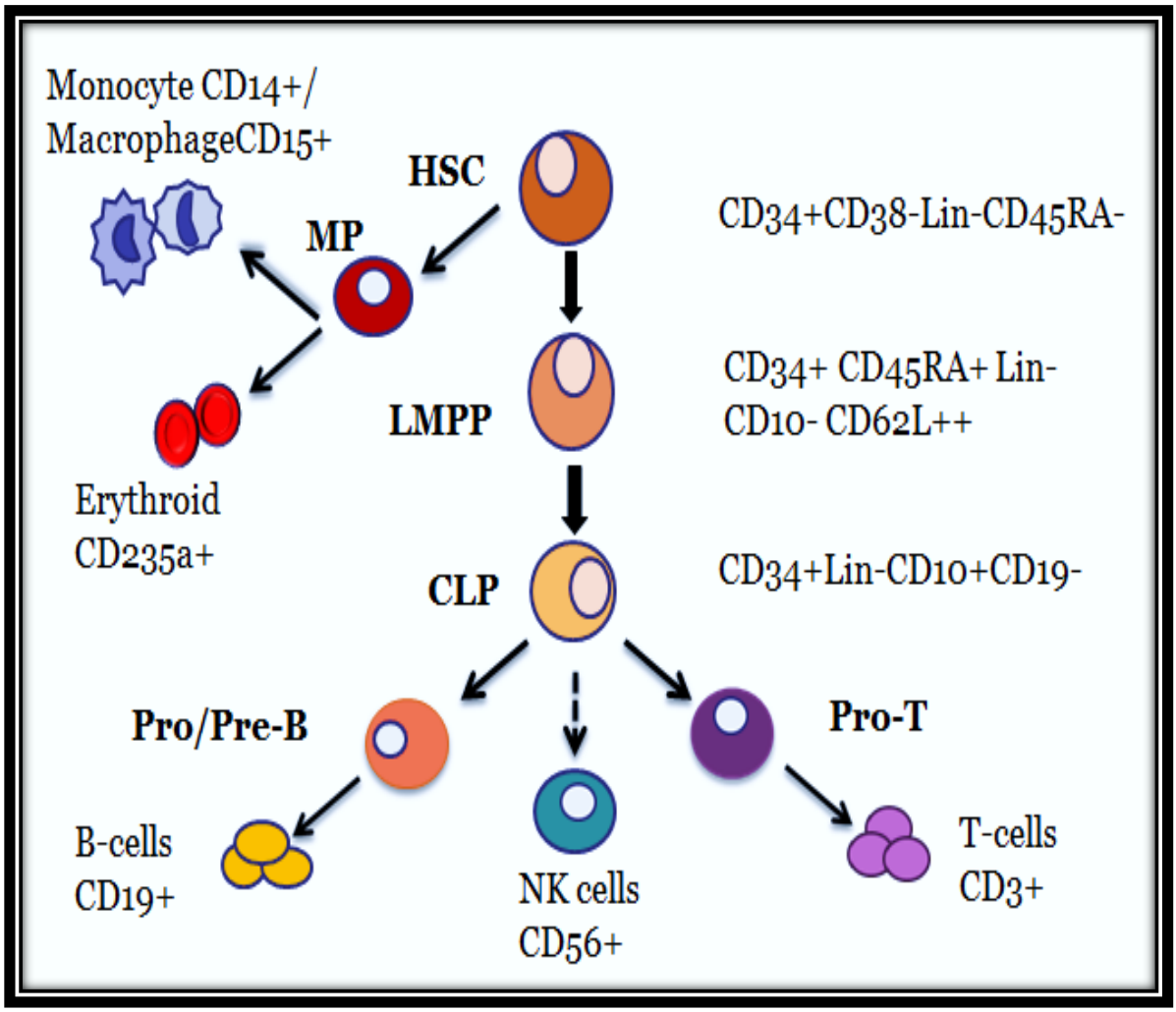

Fig. 2: Overview of progression in early hematopoietic stem cells to mature lymphoid progenitors.
HSCs are early hematopoietic stem cells consisting of full hematopoietic potential and give rise to all other progenitor cells. LMPPs are earliest multipotent lymphoid progenitors capable of giving rise to B-cells, T-cells, Natural Killer aka NK cells and possess some myeloid potential. CLPs are committed lymphoid progenitors that give rise to B-cells and NK cells. Pre-B progenitors are precursor cells that are capable of giving rise to only mature specialized B-cells.

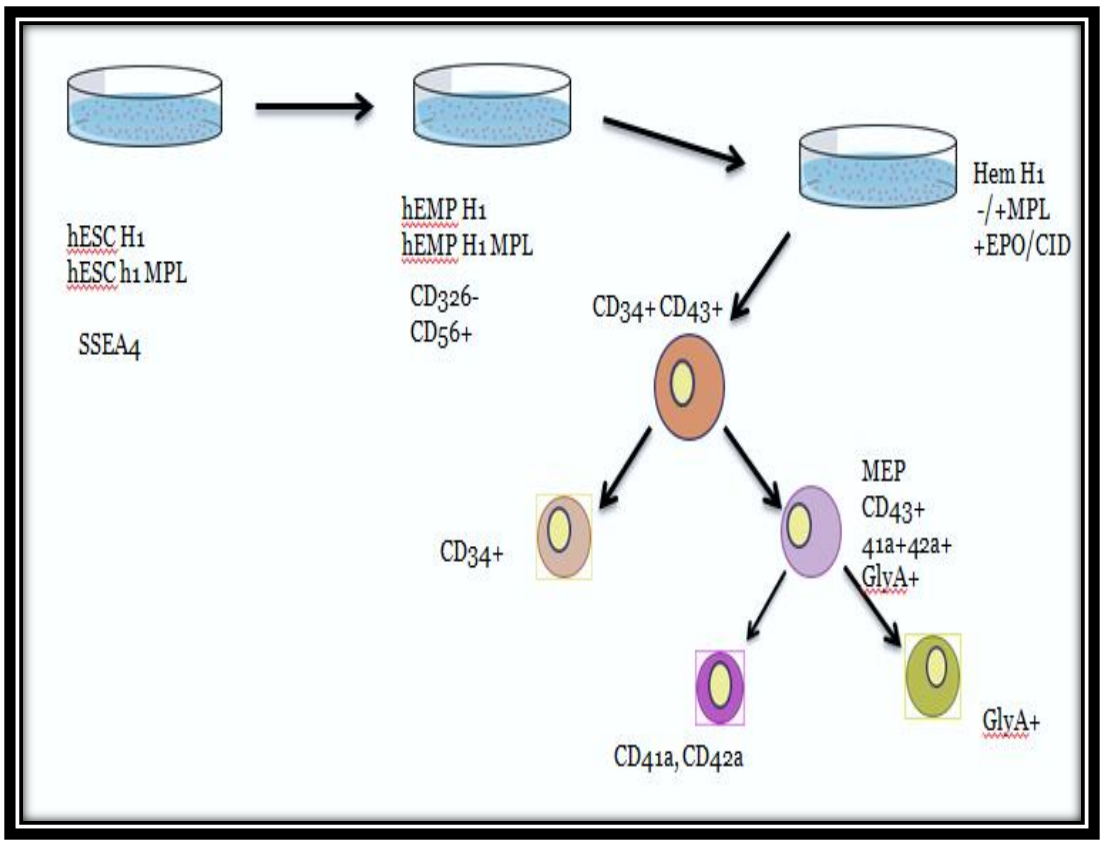

Figure 3. Schematic diagram illustrating human embryonic stem cells in culture giving rise to primitive hematopoietic stem cells, hematopoietic progenitors and differentiated cells.

Human embryonic stem cell (line H1), hESCs (SSEA4+) undifferentiated cells; hESC H1 -MPL untransduced and hESC H1 +MPL (vector) GFP+ transduced. Human embryonic mesodermal progenitors, hEMP (CD236-/CD56+; hEMP H1 untransduced, hEMP H1 MPL transduced. Hematopoietic connective tissue cells, Hem (CD34+/CD33-/CD38-/CD43+); Hem H1 untransduced, Hem H1 transduced +MPL, Hem H1 transduced +EPO (erythropoietin factor), Hem H1 transduced +CID. HSC H1 (CD34+/CD43+) early hematopoietic cells untransduced, transduced +MPL, transduced +EPO, transduced +CID. CD34+ hematopoietic stem cells lack myeloid potential; Megakaryocyte Erythroid Progenitors (MEP) CD43+/CD41a+/CD42a+/GlyA+; Megakaryocytes (CD41a, CD42a) and Erythroid cells (CD235a+).

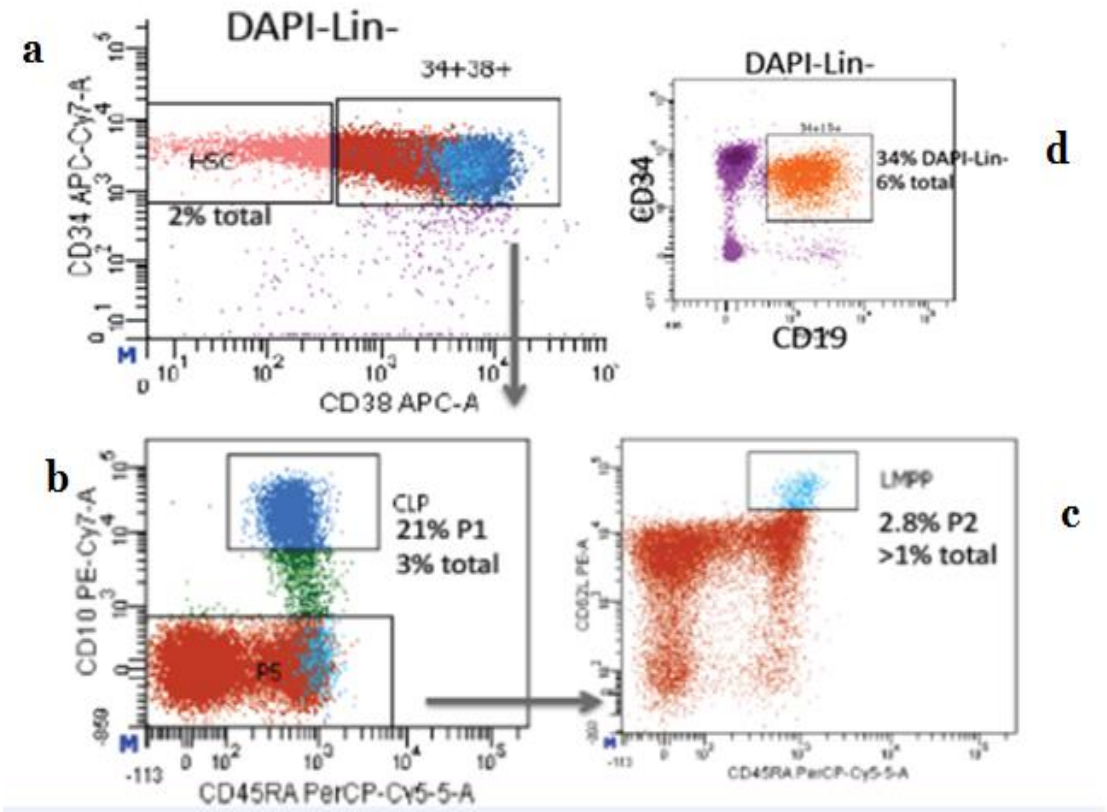

Figure 4. Flow cytometry (FACS) of the stem and progenitor cells from fresh human bone marrow <48hrs. (a). Gating strategy for collecting DAPI-/Lin-(CD3, CD14, 15, 19, 56, 57, &235a)/Lin-/34+/38- HSC cells. 34+/38+ cells are used as reference to collect progenitor cells as seen in the next panel. **(b)** Common Lymphoid Progenitor (CLP)cells are collected from the gating on DAPI-/Lin-/ CD34+/CD38+/CD10+. **(c)** Lymphoid Multipotent Progenitors (LMPP) are sorted and collected based on the gating on DAPI-/Lin-/CD34-/CD38+/CD10- as seen in the figure above.
(d) Pro-B progenitor cells sort

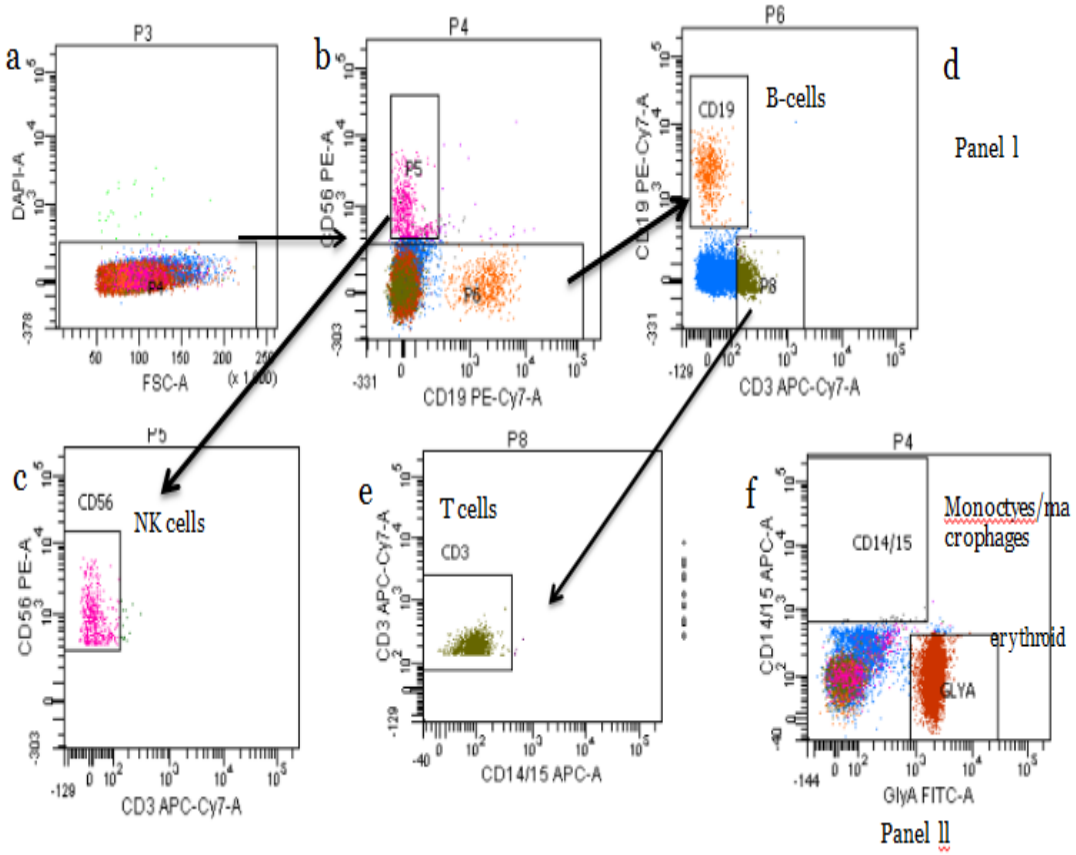

Figure 5. Gating Strategy using Flow cytometry (FACS) to sort differentiated hematopoietic cell subpopulations from fresh human bone marrow donor (<48hrs). (a) P3 shows CD34-/DAPI-/ cells which are selected and gated upon (P4) CD56+/CD19- to sort and collect Natural Killer aka NK cells CD56+/CD19-/CD3+ (Gate P5). P6 is gated upon CD34-/DAPI-/CD56-/CD19+ to sort and collect B-cells. From gating on P8 from P6, CD56-/CD19+/CD3+ T-cells were sorted and collected. (b) A separate Panel II gate (P4) was set to sort out CD34-/DAPI-/CD56-/CD14+/CD15+ Monocytes & Macrophages. CD34-/DAPI-/CD14-/CD15-/CD235a+ or GlycophorinA+ (GLYA+) erythroid cells were collected as well as seen in the figure above.

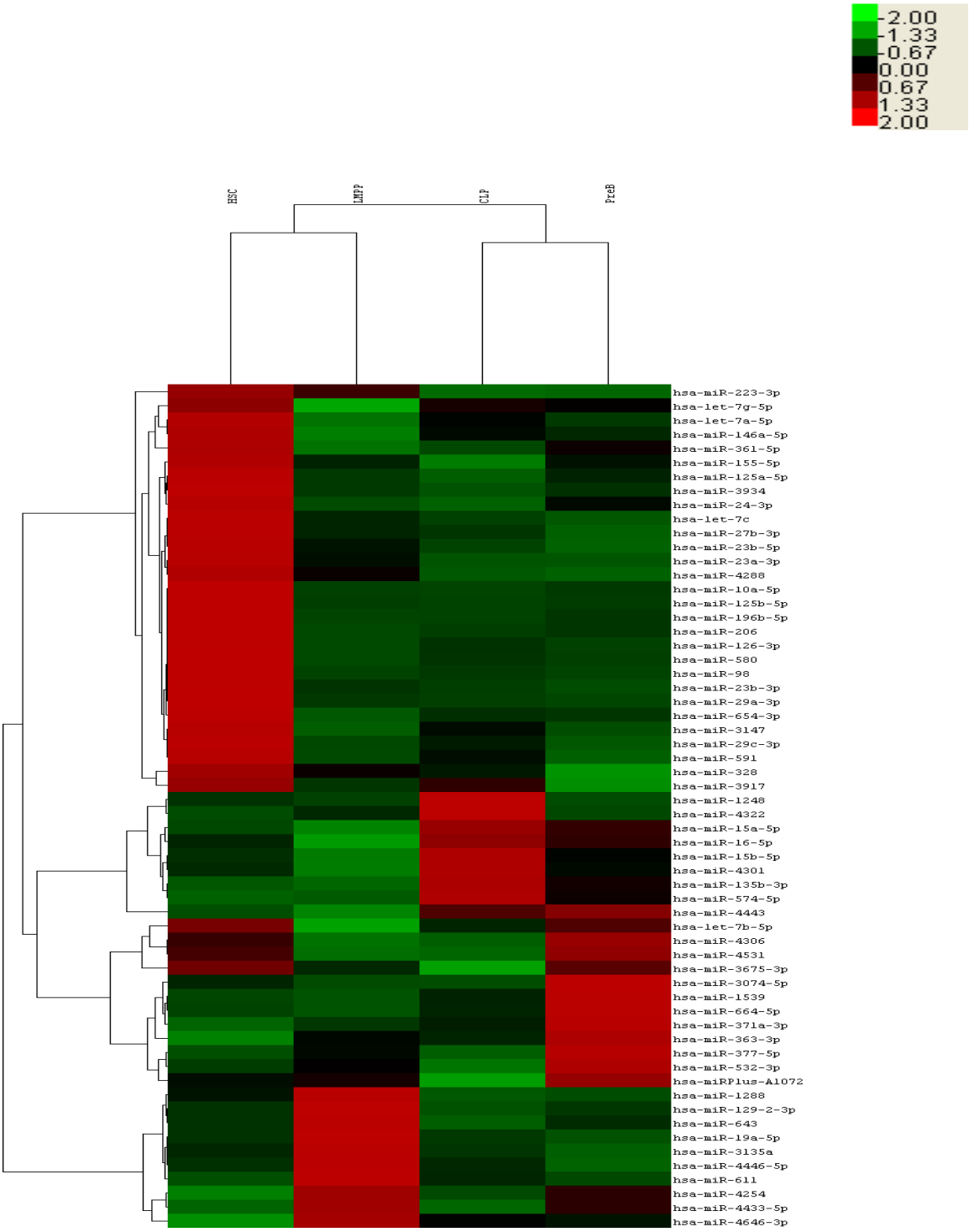

Figure 6: Hierarchical Clustering of miRNA expression profile

Heat maps of the differential expression of microRNAs in Hematopoietic Stem Cells (HSC), Lymphoid-Primed Multipotent Progenitors (LMPP), Common Lymphoid Progenitors (CLP), and Precursor B-cells (Pre-B) cell populations from Human Bone Marrow. Red represents the up regulated miRNAs, 2.0 fold increase or higher and green represents negatively regulated miRNAs by at least 2.0 fold reduction in expression levels.

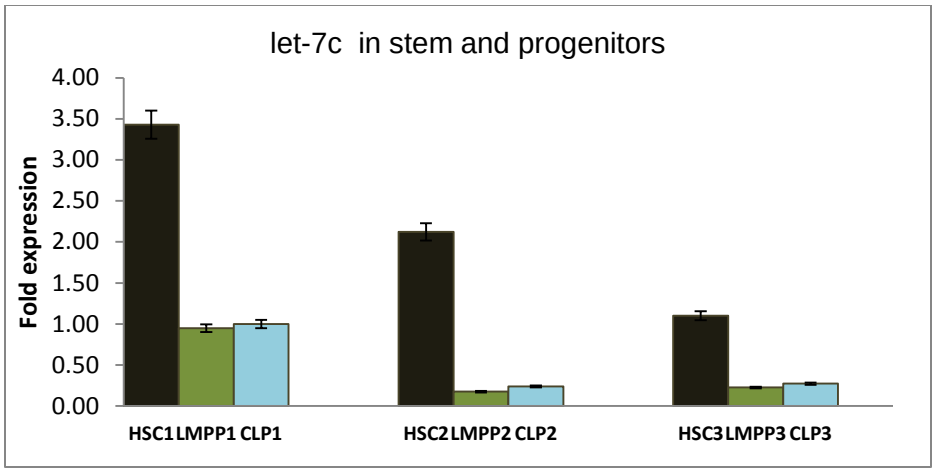

Figure 7. Quantitative analysis of let-7c expression in stem and progenitor cells from fresh bone marrow samples.
Data is represented as the relative fold increase in level of expression of miRNAs let-7cin Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2, &3), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-CD10-/CD62Lhi (LMPP 1, 2, & 3), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2, & 3) cell populations from biological samples (n=3) in technical replicates (n=3) as the miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) in various populations.

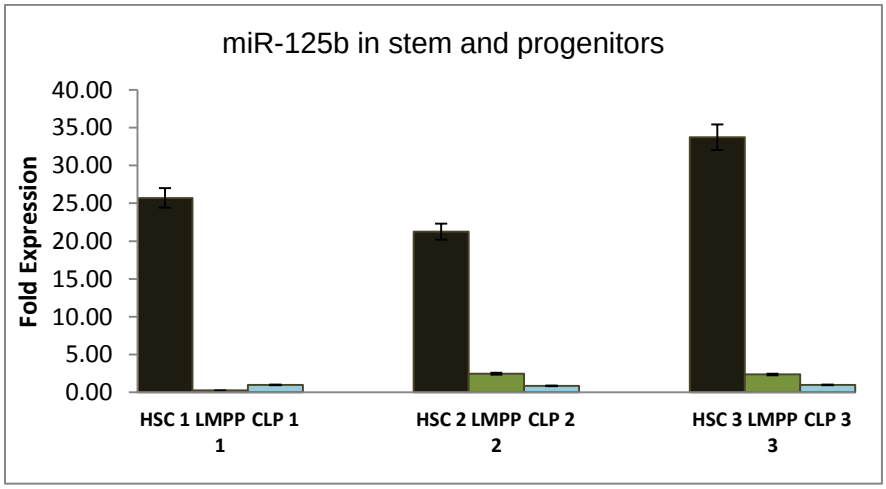

Figure 8: Quantitative analysis of miR-125b expression in stem and progenitor cells from fresh bone marrow samples.
Data is represented as the relative fold increase in level of expression of miR-125b in Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2, &3), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-CD10-/CD62Lhi (LMPP 1, 2, & 3), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2, & 3) cell populations from biological samples (n=3) in technical replicates (n=3) as the miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) in various populations

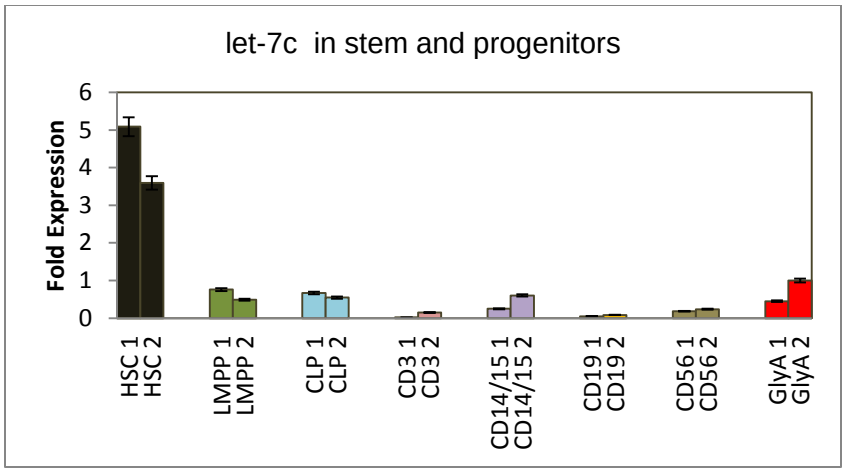

Figure 9: Quantitative analysis of let-7c expression in stem, progenitors, and differentiated cells from fresh bone marrow samples.
Data is represented as the fold increase in expression level of miRNA let-7c in Hematopoietic stem cells CD34+/CD38-/Lin- (HSCs), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPPs) and Common Lymphoid Progenitors CD34+/CD10-(CLPs); differentiated (CD34-)cell subpopulations- CD3+ (T-cells), CD14+/CD15+ (Monocytes/Macrophages), CD19+ (B-cells), CD56+ (Natural Killer, NK) cells, and CD235a+ (aka Glycophroin A or GlyA+) erythroid cell populations from biological samples (n=2) in technical replicates (n=3) as the miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) in various populations.

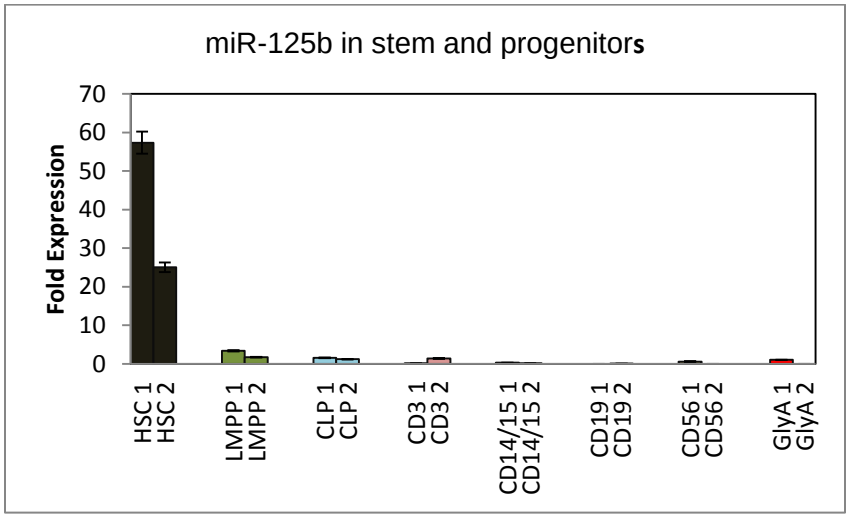

Figure 10: Quantitative analysis of miR-125b expression in stem, progenitors, and differentiated cells from fresh bone marrow samples.
Data is represented as the fold increase in level of expression of miR-99a in Hematopoietic stem cells CD34+/CD38-/Lin- (HSCs), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPPs), Common Lymphoid Progenitors CD34+/CD10-(CLPs), differentiated (CD34-)cell subpopulations- CD3+ (T-cells), CD14+/CD15+ (Monocytes/Macrophages), CD19+ (B-cells), CD56+ (Natural Killer, NK) cells, and CD235a+ (aka Glycophroin A or GlyA+) erythroid cell populations from biological samples (n=2) in technical replicates (n=3) as the miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) in various populations.

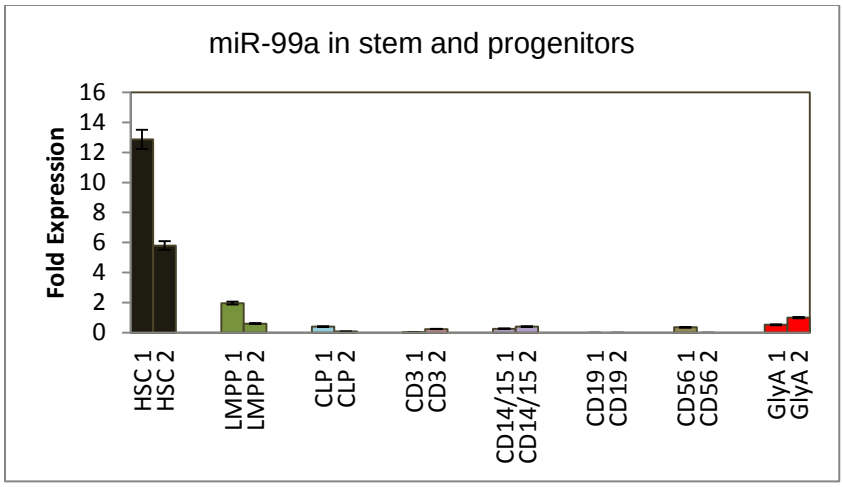

Figure 11: Quantitative analysis of miR-99a expression in stem, progenitors, and differentiated cells from fresh bone marrow samples.
Data is represented as the fold increase in level of expression of miR-99a in Hematopoietic stem cells CD34+/CD38-/Lin- (HSCs), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPPs), Common Lymphoid Progenitors CD34+/CD10-(CLPs), and differentiated (CD34-) cell subpopulations- CD3+ (T-cells), CD14+/CD15+ (Monocytes/Macrophages), CD19+ (B-cells), CD56+ (Natural Killer aka NK cells), and CD235a+ or GlycophorinA+(Erythroid cells) from biological samples (n=2) in technical replicates (n=3) as the miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) in various populations.

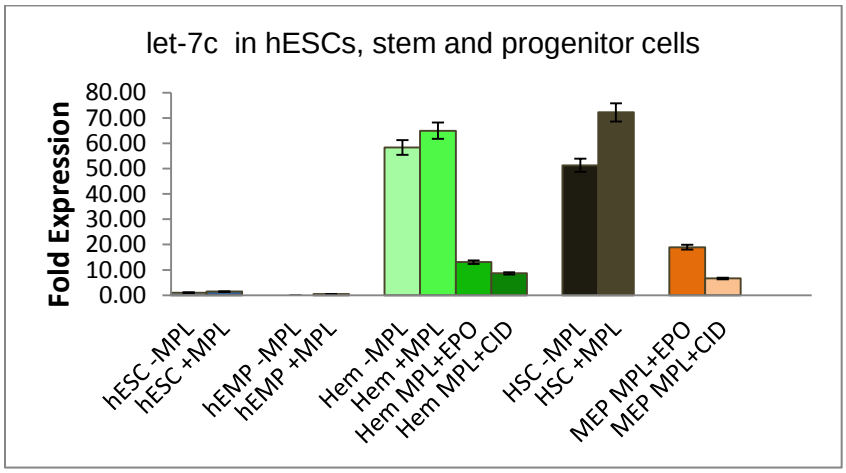

Figure 12. Quantitative PCR analysis of let-7c expression in undifferentiated embryonic hESC stem, earliest hematopoietic stem cells and progenitor MEP cells.
Expression let-7c in earliest embryonic undifferentiated, non-hematopoietic stem cells hESCs (SSEA4+), and human embryonic mesodermal progenitors, hEMP (CD236-/CD56+); early primitive form of Hematopoietic stem cells, undifferentiated and lacking myeloid potential, Hem CD34+/CD33-/CD38-/CD43+ (Hem-MPL, Hem+MPL) and in hematopoietic stem cells, HSC CD34+/CD43+ (HSC-MPL, HSC+MPL; Megakaryocyte-Erythroid potential, MEPs CD43+/CD41a+/CD42a+/GlyA+. Results are

representative of cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify fold increase expression in various cell populations.

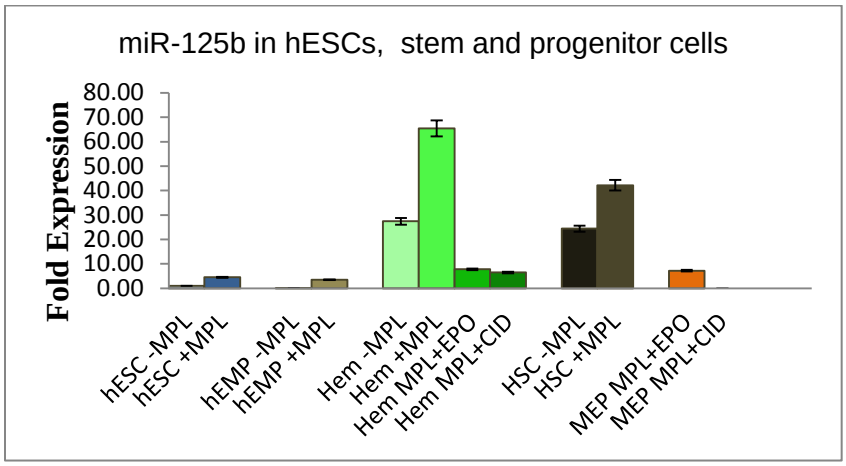

Figure 13. Quantitative PCR analysis of miR-125b expression in hESC stem, hematopoietic stem and progenitor MEP cells.
Expression of miR-125b in early primitive form of Hematopoietic stem cells, undifferentiated and lacking myeloid potential, Hem CD34+/CD33-/CD38-/CD43+ (Hem-MPL, Hem+MPL) and in hematopoietic stem cells, HSC CD34+/CD43+ (HSC-MPL, HSC+MPL); earliest embryonic undifferentiated, non-hematopoietic stem cells hESCs (SSEA4+), human embryonic mesodermal progenitors, hEMP CD236-/CD56+, and also in Hem erythroid driven vector MPL transduced cells in presence of erythropoietin factors, EPO/CID; Megakaryocyte-Erythroid potential, MEPs CD43+/CD41a+/CD42a+/GlyA+. Results are representative of cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold increase expression in various cell populations.

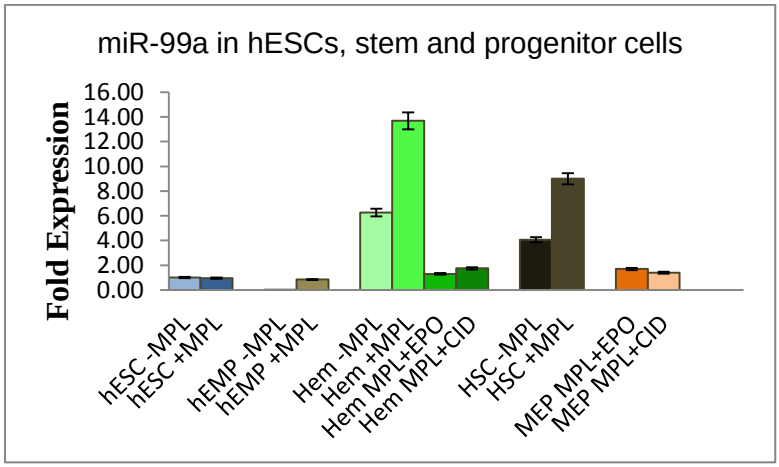

Figure 14. Quantitative PCR analysis of miR-99a expression in hESC stem, hematopoietic stem and progenitor MEP cells
Expression of miR-99a in early primitive form of Hematopoietic stem cells, undifferentiated and lacking myeloid potential, Hem CD34+/CD33-/CD38-/CD43+ (Hem-MPL, Hem+MPL) and in hematopoietic stem cells, HSC CD34+/CD43+ (HSC-MPL, HSC+MPL); Earliest embryonic undifferentiated, non-hematopoietic stem cells, hESCs (SSEA4+), human embryonic mesodermal progenitors, hEMP CD236-

/CD56+, and also in Hem erythroid driven vector MPL transduced cells in presence of erythropoietin factors, EPO/CID; Megakaryocyte-Erythroid potential, MEPs CD43+/CD41a+/CD42a+/GlyA+. Results are representative of cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold increase expression in various populations.

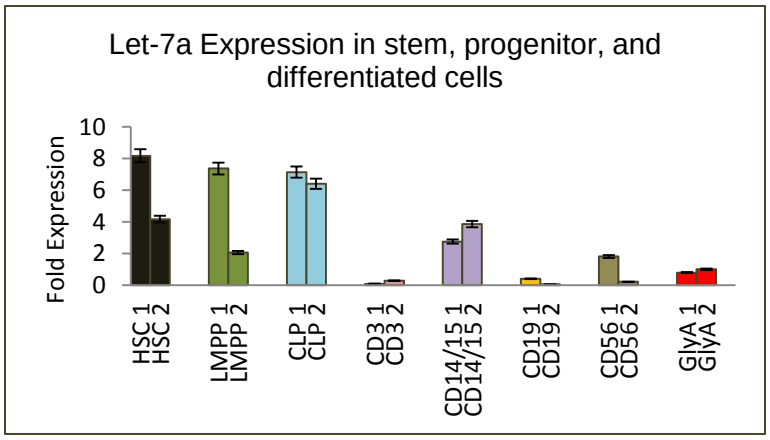

Figure 15a. Quantitative analysis of let-7a expression in stem, progenitor and differentiated cells from bone marrow samples.
Y axis represents the fold increase in level of expression of lt-7a in Hematopoietic stem cells CD34+/CD38-/Lin- (HSCs), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPPs), Common Lymphoid Progenitors CD34+/CD10-(CLPs), differentiated (CD34-)cell populations CD3+ (T-cells), CD14+/CD15+ (Monocytes/Macrophages), CD19+ (B-cells), CD56+ (Natural Killer, NK) cells, and CD235a+ (aka Glycophroin A or GlyA+) erythroid cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold increase expression in various populations.

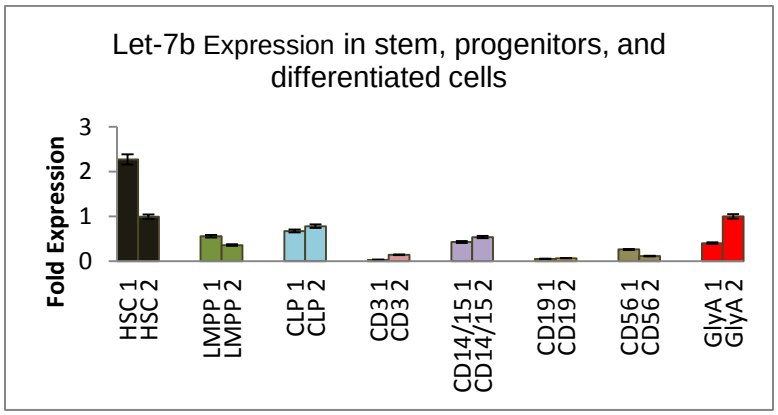

Figure 15b. Quantitative analysis of let-7b expression in stem, progenitor and differentiated cells from bone marrow samples.

Y axis represents the fold increase in level of expression of let-7b in Hematopoietic stem cells CD34+/CD38-/Lin- (HSCs), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPPs), Common Lymphoid Progenitors CD34+/CD10-(CLPs), differentiated (CD34-)cell populations CD3+ (T-cells), CD14+/CD15+ (Monocytes/Macrophages), CD19+ (B-cells), CD56+ (Natural Killer, NK)

cells, and CD235a+ (aka Glycophroin A or GlyA+) erythroid cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify fold increase expression in various populations.

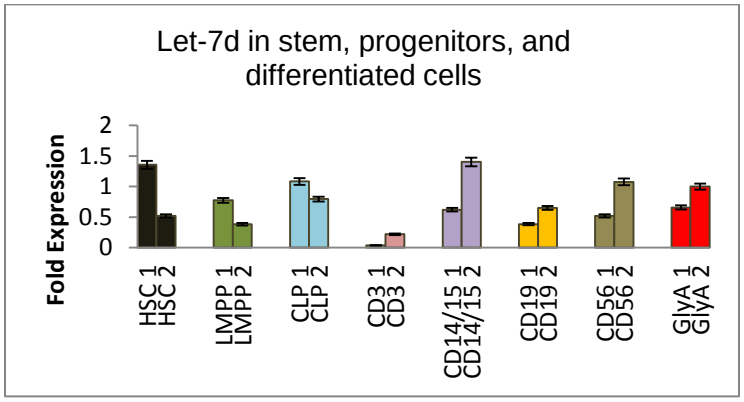

Figure 15c. Quantitative analysis of let-7d expression in stem, progenitor and differentiated cells from bone marrow samples.
Y axis represents the fold increase in level of expression of let-7d in Hematopoietic stem cells CD34+/CD38-/Lin- (HSCs), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPPs), Common Lymphoid Progenitors CD34+/CD10-(CLPs), differentiated (CD34-)cell populations CD3+ (T-cells), CD14+/CD15+ (Monocytes/Macrophages), CD19+ (B-cells), CD56+ (Natural Killer, NK) cells, and CD235a+ (aka Glycophroin A or GlyA+) erythroid cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify fold increase expression in various populations.

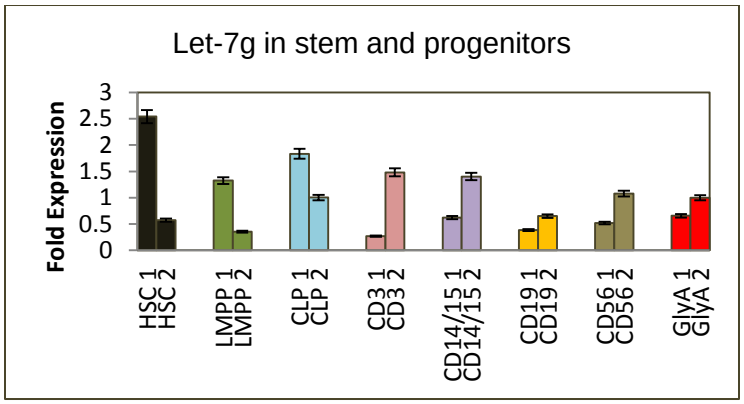

Figure 15d. Quantitative analysis of let-7g expression in stem, progenitor and differentiated cells from bone marrow samples.
Y axis represents the fold increase in level of expression of let-7g in Hematopoietic stem cells CD34+/CD38-/Lin- (HSCs), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPPs), Common Lymphoid Progenitors CD34+/CD10-(CLPs), differentiated (CD34-)cell populations CD3+ (T-cells), CD14+/CD15+ (Monocytes/Macrophages), CD19+ (B-cells), CD56+ (Natural Killer, NK) cells, and CD235a+ (aka Glycophroin A or GlyA+) erythroid cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify fold increase expression in various populations.

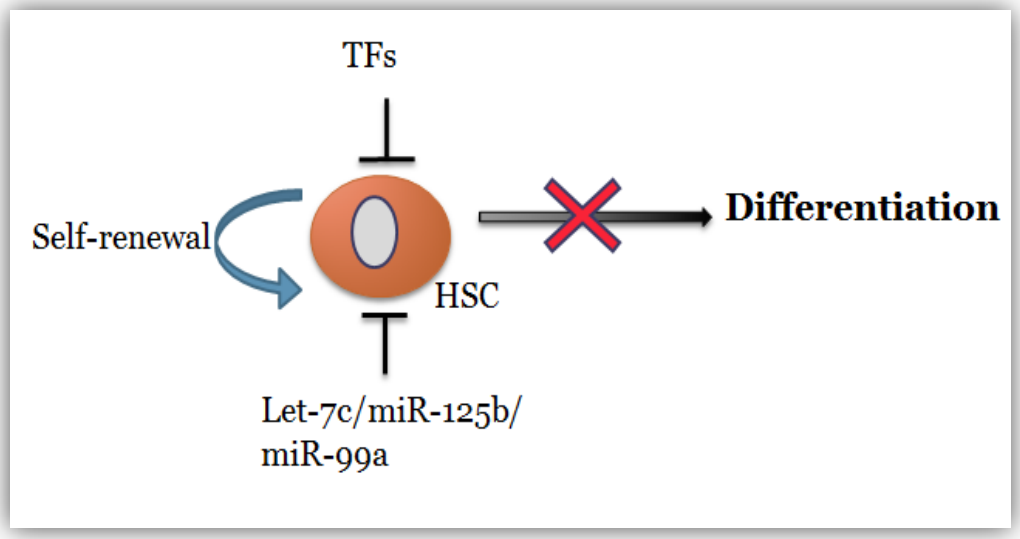

Figure 16. Proposed role of let-7c/miR-125b/miR-99a in the Hematopoietic development

Let-7c/mir-125b/mir-99a may be co-regulators along with other Transcription factors (TFs) and involved at the earliest level of hematopoietic development in the human bone marrow based on their expression pattern in hematopoietic stem cells, HSCs.

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APPENDIX A

Supplementary Figure 1: List of Taqman miRNA primer probes (Life Technologies)

microRNA name	**Assay Id**
hsa-miR-10a	000387
hsa-miR-125b	000449
hsa-miR-223-3p	002295
hsa-miR-24-3p	000402
hsa-miR-4288	242342
hsa-miR-29a	002112
hsa-miR-580	001621
hsa-miR-654-3p	002239
hsa-miR-3147	243939
hsa-miR-146a-5p	000468
hsa-miR-99a	000435
Let-7a	000377
Let-7b	002619
Let-7c	000379
Let-7d	002283
Let-7g	002282
hsa-miR-1288	002832
hsa-miR-129-3p	001184
hsa-miR-643	001594
hsa-miR-19a-5p	002424
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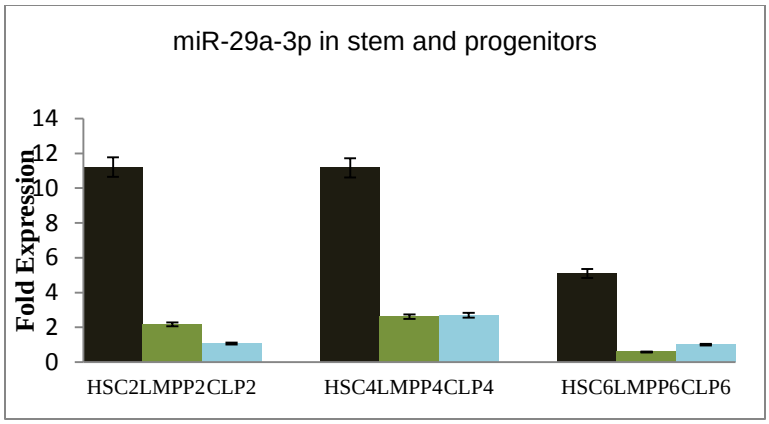
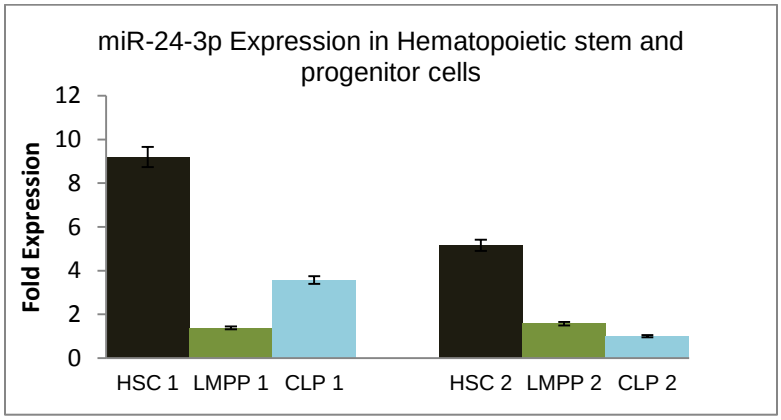
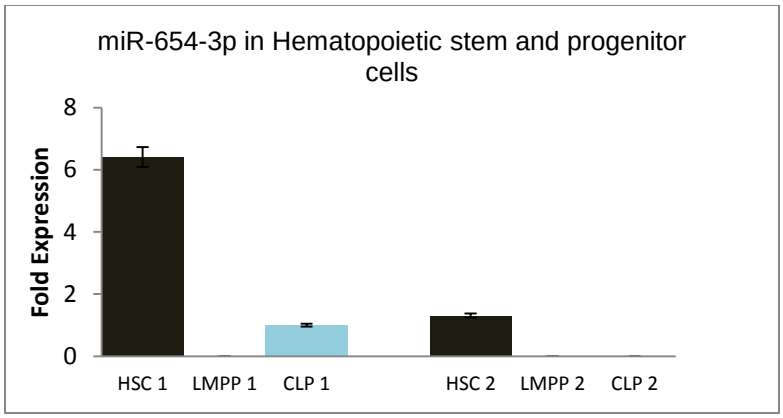
hsa-miR-4322	243221
hsa-miR-135b-3p	002261
RNU44	001094
RNU48	001006



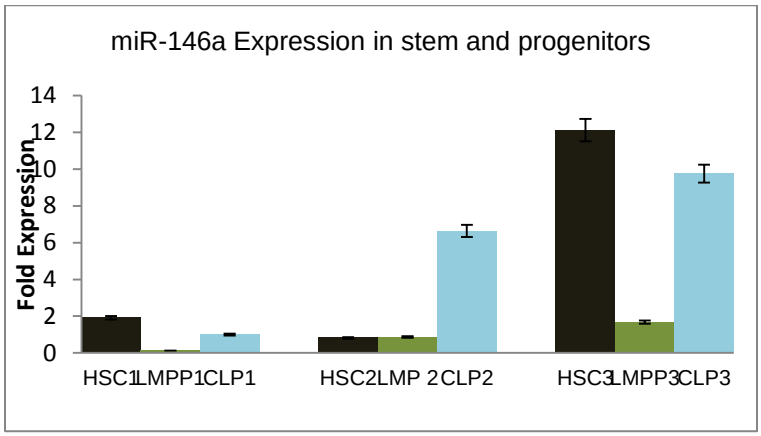
Supplementary Figure 2. Quantitative analysis of miR-29a-3p expression in stem and progenitors from fresh bone marrow samples.
Y axis represents the relative fold increase in level of expression of miR-29a-3p in Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2, &3), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPP 1, 2, & 3), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2, & 3) cell populations from biological samples (n=3) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold expression in various populations



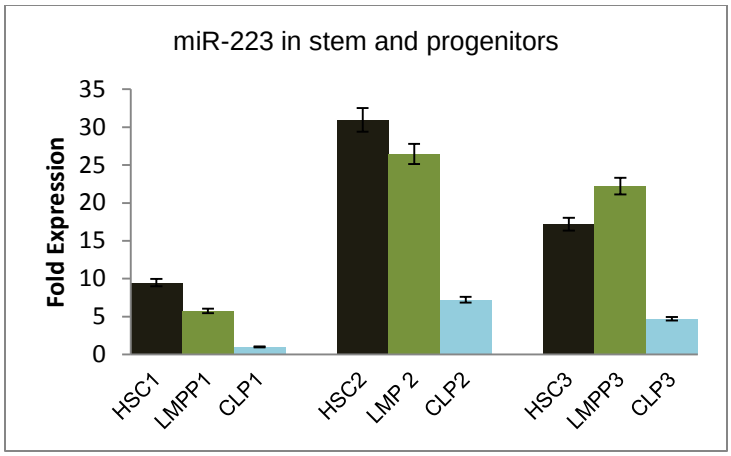
Supplementary Figure 3. Quantitative analysis of miR-24-3p expression in stem and progenitors from fresh bone marrow samples.
Y axis represents the relative fold increase in level of expression of miR-24-3p in Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPP 1, 2), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2) cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold expression in various populations.



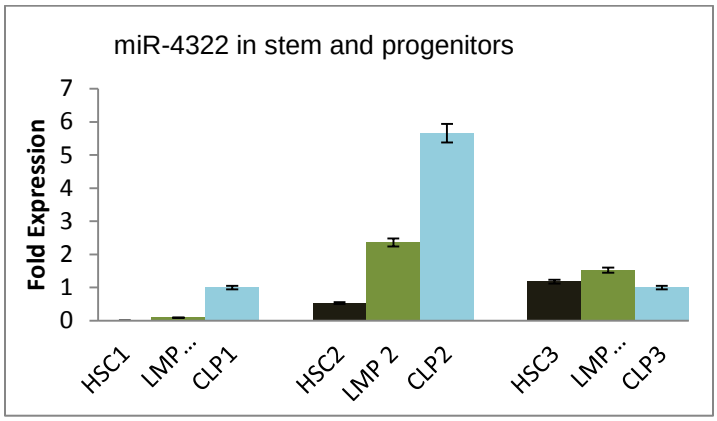
Supplementary Figure 4. Quantitative analysis of miR-654-3p expression in stem and progenitors from fresh bone marrow samples.
Y axis represents the relative fold increase in level of expression of miR-654-3p in Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPP 1, 2), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2) cell populations from biological samples (n=2) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold expression in various populations.



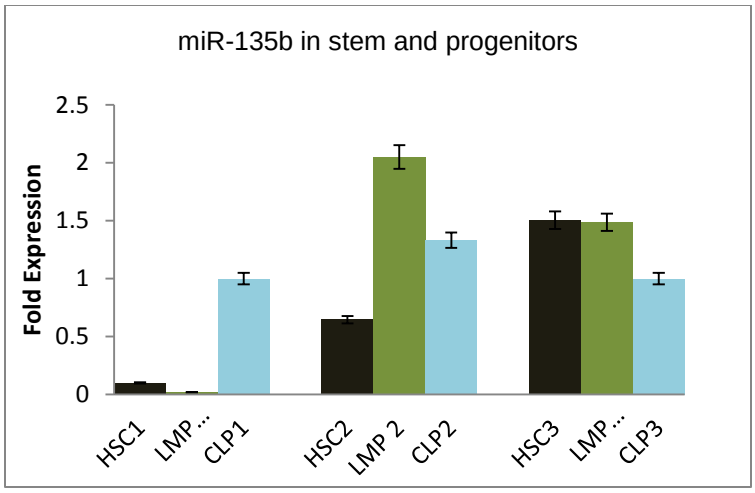
Supplementary Figure 5. Quantitative analysis of miR-146a expression in stem and progenitors from fresh bone marrow samples.
Y axis represents the relative fold increase in level of expression of miR-146a in Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2, &3), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPP 1, 2, & 3), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2, & 3) cell populations from biological samples (n=3) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold expression in various populations.



Supplementary Figure 6. Quantitative analysis of miR-223 expression in stem and progenitors from fresh bone marrow samples.
Y axis represents the relative fold increase in level of expression of miR-223 in Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2, &3), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPP 1, 2, & 3), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2, & 3) cell populations from biological samples (n=3) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold expression in various populations.



Supplementary Figure 7. Quantitative analysis of miR-4322 expression in stem and progenitors from fresh bone marrow samples.
Y axis represents the relative fold increase in level of expression of miR-4322 in Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2, &3), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPP 1, 2, & 3), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2, & 3) cell populations from biological samples (n=3) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify relative fold expression in various populations.



Supplementary Figure 8. Quantitative analysis of miR-135b expression in stem and progenitors from fresh bone marrow samples.
Y axis represents the relative fold increase in level of expression of miR-135b in Hematopoietic stem cells CD34+/CD38-/Lin- (HSC 1, 2, &3), Lymphoid-Primed Multipotent Progenitors CD34+/CD38-/CD10-/CD62Lhi (LMPP 1, 2, & 3), and Common Lymphoid Progenitors CD34+/CD10-(CLP 1, 2, & 3) cell populations from biological samples (n=3) in technical replicates (n=3). The miRNA gene expression levels are normalized to the housekeeping gene RNU44 (endogenous control) to quantify fold expression in various populations.